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by

© Brian Campbell Foster

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Dedication

To my mother,

for her love and support.

Abstract

The objectives of the present study were three fold:

1. To identify good examples of microbial models of drug metabolism;
2. To identify good examples of micro-organisms capable of difficult chemical synthesis; and
3. To optimize the techniques used in microbial syntheses.

To establish the feasibility of using microbial models of mammalian drug metabolism required the examination of a series of structurally related compounds. This has been accomplished by studying the metabolism of amphetamine-like compounds. With the large number of amphetamine like compounds which are pharmacologically interesting, it was necessary to restrict this study to three subgroups of the amphetamines, namely

1. The phenylethylamines, amphetamines and phentermines;
2. Hydrazine derivatives of these compounds; and
3. Tertiary amines with a propynylic functionality.

In addition, the metabolism of fluphenazine was also examined for S-oxidation. N-(*n*-Propyl)amphetamine (NPA) was selected as the initial substrate because it possessed a simple structure and yet was capable of undergoing a multiplicity of biological transformations.

Extracts of incubation broths containing micro-organisms, drug substrates and their metabolites, and control broths to which substrates but no micro-organisms had been added were examined by gas-liquid chromatography

(GLC). Identification of metabolites was generally based on comparison of their GLC retention times and mass spectra with those of authentic reference compounds. In some instances, identification of some metabolites or their chemical derivatives was based on interpretation of mass spectral fragmentation patterns.

The organisms were chosen from a wide variety of bacteria and fungi which were used initially to metabolize the model compound NPA. Two micro-organisms, *Cunninghamella echinulata* and *Mycobacterium smegmatis* were selected by routine screening methods for their ability to N-oxygenate NPA. Biotransformation studies with *C. echinulata* revealed that this organism is an excellent microbial model of mammalian metabolism as this organism was able to mediate N-dealkylation, oxidative deamination, N-acetylation, ring hydroxylation, and N- and S-oxidation of numerous substrates in a manner similar to that observed in mammalian drug metabolism systems. *M. smegmatis* was found to mediate the biotransformation of numerous substrates through N-oxidation, N-dealkylation, oxidative deamination and N-acetylation.

Immobilized and pelleted cultures of *C. echinulata* and *M. smegmatis* displayed characteristics different from those of freely suspended cells. The useable life of the microbial incubation mixtures was prolonged and the development of bubble reactors increased the usefulness of these micro-organisms for the synthesis of important products.

Further development of the bioreactors resulted in the design of a air-lift reactor with an external loop which enhanced the biotransformation capabilities of these organisms.

QUOTATION

Whenever a new discovery is reported to the scientific world, they say first, "It is probably not true." Thereafter when the truth of a new proposition has been demonstrated beyond question, they say, "Yes, it may be true, but it is not important." Finally, when sufficient time has elapsed to fully evidence its importance, they say, "Yes, surely it is important, but it is no longer new."

Michel DeMontaigne

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I wish to acknowledge the graduate students in this department. They made the past few years interesting and enjoyable.

My family and friends cannot be thanked enough for their support during my days as a graduate student.

Abbreviations and Symbols

ABF, *alpha*-methyl-N-(1-methylacetonylidene)benzeneethanamine

ABG, *alpha*-methyl-N-(1-methylacetonyl)benzeneethanamine

ABH, *alpha*-methyl-N-(1-methyl-2-hydroxypropyl)benzeneethanamine

AcAMPH, N-acetylamphetamine

AcBEN, N-acetylbenzylamine

AcCLA, N-acetyl-*p*-chloroamphetamine

AcCLE, N-acetyl-*p*-chlorophenylethylamine

AcFAM, 2-acetylamino-3-fluoro-1-phenylpropane

AcPHE, N-acetylphenylethylamine

AcPLZ, 1-acetyl-2-(2-phenylethyl)hydrazine

AcTCA, N-acetyl-2-phenylcyclopropanamine

AMPH, amphetamine

AOO, 2-nitroso-1-phenylpropane

APP, 2-amino-3-phenylpropanol

ATCC, American Type Culture Collection

AZI, 2-benzylaziridine

BEN, benzylamine

β, beta

BOX, *alpha*-methyl-N-(*n*-butylidene)benzeneethanamine N-oxide

BSA, N,O-bis(trimethylsilyl)acetamide

BSTFA, N,O-bis-(trimethylsilyl)trifluoroacetamide

BTOX, *alpha,alpha*-dimethyl-N-(*n*-butylidene)benzeneethanamine N-oxide

BX, Bristol Myers Laboratories

CBB, 4-chloro-*alpha*-methyl-N-(1-methylacetonylidene)-benzeneethanamine

CBC, 4-chloro-*alpha*-methyl-N-(1-methylacetonyl)benzene-ethanamine

CBD, 4-chloro-*alpha*-methyl-N-(1-methyl-2-hydroxypropyl)-benzeneethanamine

CLA, *p*-chloroamphetamine

CLE, *p*-chlorophenylethylamine

CPA, *p*-chloro-*β*-phenylacetic acid

DCIP, 2,6-dichlorophenolindophenol

DEP, deprenyl

DPE, N,N-(di-*n*-propyl)phenylethylamine

EI-MS, electron impact mass spectrometry

EOX, *alpha*-methyl-N-(ethylidene)benzeneethanamine N-oxide

ETOX, *alpha,alpha*-dimethyl-N-(ethylidene)benzeneethan-amine N-oxide

FAM, 2-amino-3-fluoro-1-phenylpropane

FEN, N-(2-cyanoethyl)amphetamine

FLU, fluphenazine

FSX, fluphenazine sulfoxide

GLC, gas-liquid chromatography

GLC/MS, gas-liquid chromatography/mass spectrometry

*p*OH-AMPH, *p*-hydroxyamphetamine

*p*OH-IPA, *p*-hydroxy-N-(isopropyl)amphetamine

*p*OH-IPT, *p*-hydroxy-N-(isopropyl)phentermine

*p*OH-NPA, *p*-hydroxy-N-(*n*-propyl)amphetamine

*p*OH-NPH, *p*-hydroxy-N-(*n*-propyl)phentermine

*p*OH-PHT, *p*-hydroxyphentermine

HMC, 2-hydroxy-N-(*alpha*-methylphenylethyl)-azetidine

HPA, N-(2-hydroxy-*n*-propyl)amphetamine

IAM, 2-amino-3-iodo-1-phenylpropane

IOH, N-hydroxy-N-(isopropyl)amphetamine

IOX, *alpha*-methyl-N-(isopropylidene)benzeneethanamine N-oxide

IPA, N-(isopropyl)amphetamine

IPE, N-(isopropyl)phenylethylamine

IPT, N-(isopropyl)phentermine

ITOH, N-hydroxy-N-(isopropyl)phentermine

MAO, monoamine oxidase

MAOI, monoamine oxidase inhibitor

MBA, N-methylbenzylamine

MC, 3-methylcholanthrene

MCP, N-(*alpha*-methylphenylethyl)-azetidine

MEF, N-(3-chloropropyl)amphetamine

MOX, *alpha*-methyl-N-(methylene)benzeneethanamine N-oxide

MS, mass spectrometry

NBA, N-(*n*-butyl)amphetamine

NBT, N-(*n*-butyl)phentermine

NCIB, National Collection of Industrial Bacteria

NEA, N-(ethyl)amphetamine

NET, N-(ethyl)phentermine

NMA, N-methylamphetamine

NMT, N-methylphentermine

NOH-AMPH, N-hydroxyamphetamine

NOH-PBA, N-hydroxy-N-propargylbenzylamine

NOH-IPA, N-hydroxy-N-(isopropyl)amphetamine

NOH-NBA, N-hydroxy-N-(*n*-butyl)amphetamine

NOH-NEA, N-hydroxy-N-(ethyl)amphetamine

NOH-NPA, N-hydroxy-N-(*n*-propyl)amphetamine

NOH-NPT, N-hydroxy-N-(*n*-propyl)phentermine

7OH-FLU, 7-hydroxyfluphenazine

NOX, *alpha*-methyl-N-(*n*-propylidene)benzeneethanamine N-oxide

NTOX, *alpha,alpha*-dimethyl-N-(*n*-propylidene)benzeneethanamine N-oxide

NPA, N-(*n*-propyl)amphetamine

NPE, N-(*n*-propyl)phenylethylamine

NPT, N-(*n*-propyl)phentermine

OXM, 1-phenyl-2-propanone oxime

PAA, phenylacetic acid

PAR, pargyline

PB, phenobarbital

PBA, N-propargylbenzylamine

PHE, phenylethylamine

PHT, phentermine

PLZ, phenelzine

PNZ, pheniprazine

PRL, Prairie Regional Laboratory

SKF-525A, diethylaminoethyl-2,2-diphenylvalerate HCl

TCA, 2-phenylcyclopropanamine

TFA, trifluoracetyl

TFAA, trifluoroacetic anhydride

TLC, thin-layer chromatography

TOH, N-hydroxyphentermine

TON, *alpha,alpha*-dimethyl-*alpha*-nitro-β-phenylethane

TOO, *alpha,alpha* dimethyl-*alpha*-nitroso-β-phenylethane

TSB, trypticase soy broth

UAH, University of Alberta Hospital

UAMH, University of Alberta Mold Herbarium and Culture Collection

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1. Introduction

All living organisms are being constantly challenged by an ever increasing number of foreign organic and inorganic compounds. The American Chemical Society's Chemical Abstract Registry contained over four million distinct chemical entities up to November 1977. This list is increasing at an average rate of about 6000 compounds per week. Current estimates are that 63,000 chemicals are in common use; about 12,000 of these are used in the food and pharmaceutical industries (Blumberg 1978).

Many of these compounds are foreign to the normal metabolic pathways of an organism. It is essential then to study the biotransformation of at least representative classes of these compounds to determine whether a substrate is activated or inactivated during the metabolic process. Occasionally, a compound may exhibit undesirable characteristics. For example, it may be transformed into a potent alkylating agent which can react with intracellular macromolecules capable of cellular destruction. Traditional mammalian studies are too restrictive and complicated to manage effectively the number of compounds that have to be studied.

Smith and Rosazza (1975) legitimized the concept of microbial models of mammalian drug metabolizing systems and the model has proven to be efficient for the testing of several classes of compounds. The object of the study reported in this thesis was to test the usefulness of

microbial systems in the metabolism of aryl aliphatic amines.

1.1 Drug Metabolism

Studies on the steady state concentration of drugs in mammalian systems have shown that there are marked inter-individual differences in metabolism. The differences can be attributed to both genetic and environmental factors. Most of the compounds that we are exposed to are innocuous and are metabolized or excreted as harmless products. In some instances, the compound does not evoke a biological effect until it has been metabolized. The activity of the compound may be enhanced, lost or even changed as a result of its metabolism. The excretion rate of the chemical may be altered by this transformation.

The relationship between drug metabolism and pharmacological activity was conceptualized during the latter half of the nineteenth century. Heubner (1913) expanded this knowledge with the finding that there was a correlation between aromatic amine metabolism and methemoglubinemia. It was many years later that the significance of this observation was understood. It is now known that biotransformation of medicinally important compounds may result in iatrogenic disease, blood dyscrasias, liver necrosis, allergies, anaphylactic shock, mutations, carcinomas and other toxic conditions (Drayer 1974, 1976;

Mitchell *et al*. 1973; Smuckler 1977). Disease and aging affects the drug metabolism system and changes the drug disposition and response (Reidenberger and Drayer 1978; Vesell 1978; Yaffee and Danish 1978).

Drug metabolism can, in addition to providing information on the mechanism of action and toxicity of a compound, help in the development of new drugs.

1.2 Mammalian Models of Drug Metabolism

The pre-clinical investigations of biotransformations in man are usually performed with various mammalian species. The premise is that mammals are more likely to provide better models than non-mammalian organisms. The laboratory animals used are seldom chosen on the basis of comparative anatomy, physiology or biochemical criteria but on tangible factors such as availability, convenience and cost. This has resulted in the use of a few species and strains of mouse, rat and guinea pig (*rodententia*); dog, cat and ferret (*carnovoria*); rabbit (*lagomorphia*); marmoset, squirrel monkey and rhesus monkey (*anthropoidea*) and some primates (Smith 1978). The use of these animals as models is satisfactory when species-independent affects are being investigated but problems are encountered when metabolic results are species dependant (Walker 1978).

The genetic basis of drug metabolism is well established and can be influenced by certain environmental

factors (Table 1). Species variances may arise from genetic defects in the common metabolic pathways and the prevalence of unusual metabolic reactions in one species but not another. Variations may arise from competing reactions where several metabolic pathways are available and one may predominate as a result of environmental induction, inhibition or stimulation (Parke 1978; Smith 1978). The dosage of substrate is critical as a high concentration may saturate a particular enzyme and permit the expression of an alternate pathway. This problem is compounded if the substrate concentration is too low; the enzyme involved in metabolism may not be induced.

The drug formulation may influence the bioavailability and tissue concentrations of substrates. This is particularly evident if the agent is given as a pure compound or as the clinically prescribed preparation complete with excipients which can affect the dissolution and uptake of the substrate. Dissolution is affected by the rate of absorption across the gastro-intestinal mucosa, intestinal microflora (Goldman 1978) and the solubility of substrates at physiological pH (Beckett and Cowan 1978).

The assumption that *in vivo* biotransformation will be observed in *in vitro* studies if the conditions are right and that toxic agents exhibit fewer side effects *in vitro* has prompted numerous studies with these systems. *In vitro* studies are complicated by the choice between sub-cellular preparations or whole cells, each with its own advantages

Table 1 . A partial list of variables affecting drug metabolism in experimental animals (Fraser and Dollery 1978; Vesell 1978).

A. Variables in the external environment: air exchange and composition, barometric pressure (crowding), cedar bedding, cleanliness, coprophagia, diet (food, water), gravity, hepatic microsomal enzyme induction or stimulation, handling, humidity, light cycle, noise level, temperature.
B. Variables in the internal environment: adjuvant arthritis, age, alloxan diabetes, castration and hormone therapy, circadian and seasonal variations, dehydration, disease, estrous cycle, fever, gastrointestinal function and flora, genetics, hepatic flow, malnutrition, pregnancy, sex, shock (hemorrhagic or endotoxic), stress.
C. Pharmacologic variables: drugs; acute versus chronic adminstration, biowithdrawal, presence of other drugs or food, routes of administration, volume of material injected, tolerance, vehicle, etc.

and disadvantages (Smith *et al*. 1977). Preparation and use of perfused organs, tissue homogenates and slices, isolated cells and cell preparations require a high level of technical expertise. Such preparations are expensive to maintain.

Several problems are associated with the rupture of the cellular membranes. Lysozyme and other lytic enzymes are released from their storage vesicles and may become involved in the formation of metabolic artifacts or enzyme loss. Separation of cellular constituents by centrifugation results in loss of important cofactors and disruption of metabolic sequencing or co-operative effects.

The reproducibility of these methods is variable as there is a need for uniformity in all stages of the experimental procedure from sacrifice of the animal model to the incubation protocol. Minor differences in tissue or cellular extraction, homogenation, centrifugation cofactor and hormonal levels, incubation medium and technique can alter the metabolic profile.

Detection and quantification of metabolites in mammalian models have been restricted by several inherent problems:

1. Current analytical methods are insensitive and require the development of new techniques and equipment for the detection of trace amounts of drugs and metabolites in biological media;
2. Many of the metabolites may be labile and susceptible to heat, oxygen, pH, light, solid surfaces and heavy

metals;

3. Metabolites may have low lipid solubility and are very water soluble, restricting both metabolism and extraction;
4. The metabolic profile of many drugs may contain a mixture of neutral, basic and acidic molecules;
5. The stability and partitioning characteristics of the metabolites formed may vary at different extraction pH values;
6. Qualitative and quantitative metabolic differences may occur in the numerous species of laboratory animals used in these studies - especially when relating the results to man;
7. Many metabolites are costly and difficult to synthesize chemically if they have one or more asymmetric centers;
8. Maintaining large numbers of experimental animals for drug metabolism studies requires costly facilities and can be an inherent source of disease which can infect man and contaminate experimental systems;
9. Anti-vivisectionists are concerned that animal models are been used when other models exist and that the resistance to these models is due to economic and not techical factors (Broad 1980; Feldmann 1980).

These factors emphasize that the extrapolation of data from animal models to man is difficult because laboratory animals serve only as imperfect models for what may occur in man. The perfect comparative study probably cannot be done. In

view of this generalization, several investigators have attempted to find alternate models of mammalian drug metabolizing systems. It was recently shown that a microbial transformation system can mimic known mammalian mechanisms (Rosazza 1980; Smith and Rosazza 1975).

The objectives of the present study were three fold:

1. To identify good examples of microbial models of drug metabolism;
2. To identify good examples of micro-organisms capable of difficult chemical synthesis; and
3. To optimize the techniques used in microbial syntheses.

2. Literature Survey

--- It seems plain therefore, that there is a vegetative Force in every microscopical Point of Matter, and every visible Filament of which the whole animal or vegetable Texture consists: And probably this Force extends much further--

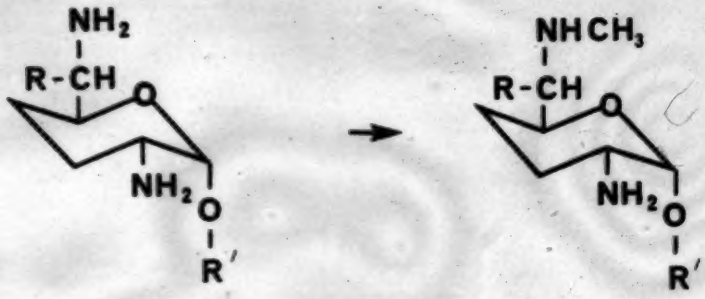
T. Needham

Phil. Trans. Roy. Soc. London (1748)

This force can now be managed for the preparation or modification of various organic compounds. Microbial enzymes have been used to develop new and innovative technologies, such as the cleavage of natural penicillins to 6-amino-penicillanic acid, the starting material for new semi-synthetic penicillins, and to replace traditional methods of coffee fermentation to enhance the yield and flavour of this product. The biotechnology of microbial bio-transformation has been successfully applied to the production of thienamycin, 5-dehydroprednisolone, actino-mycin D, lincomycin sulfoxide and a variety of other natural products. The successful use of micro-organisms in the conversion and synthesis of steroids and antibiotics demonstrated the use of these organisms as a valuable tool in synthetic organic chemistry (Beukers *et al*. 1972; Schoemer and Martin 1980; Sebek 1974; Sebek and Perlman 1971).

In addition to these multiple reaction sequences, products may also be formed by a single enzyme mediated

reaction. Typical examples are the N-methylation of gentamicin to sagamicin by *Micromonospora sagawiensis* (Scheme 1) (Okachi and Nara 1980)



Scheme 1

and stereo-selective hydroxylation of bridged bicyclic amides by *Beaveria sulfurescens* (Scheme 2) (Furstoss *et al.* 1980).

Scheme 2

The demand for new products has resulted in the concomitant formation of waste products. Microbial populations are being used efficiently in the treatment of industrial and municipal waste effluents (Dagley 1978; Faber 1979). A mixed population of micro-organisms is the main vector responsible for the degradation and detoxification of environmentally hazardous oil and chemical spills.

2.1 Microbial Models of Mammalian Metabolism

Micro-organisms have three important characteristics which make them suitable for biotransformation studies:

1. A high ratio of surface to volume which facilitates rapid uptake of nutrients and promotes increased metabolic rates.
2. A capability to mediate a wide variety of biotransformations.
3. An ability to adapt to the environment. In addition to the multi-reaction sequences , micro-organisms are capable of converting a compound into a structurally related product by the use of one or more enzymes in the cell. These bioconversions may be carried out with growing cells, resting cells, spores or dried cells (Vezina and Singh 1975).

The microbial population reacts to the presence of xenobiotics in subtle ways, depending on structural and environmental factors. Several biological devices are brought into action to facilitate the biotransformation or utilization of these compounds. These include genetic repression or induction, plasmid transfer, mutation, which may lead to the evolution of enzymes with new specificities and activities, and microbial synergism (Aharonowitz 1980; Berry 1975; Elander 1980; Johnston 1975). Commensurate with their role, the total mass of micro-organisms on this planet greatly exceeds the total mass of all animals (Dagley 1978). The most abundant micro-organisms in the biosphere are the

bacteria, but in proportion fungi account for the largest fraction of the total mass.

There is no reason why every compound must or can be degraded; in fact recalcitrant molecules do exist (Alexander 1980). Small differences in enzymatic properties and activity, membrane permeability, nutritional requirements, energy generation, temperature, aeration and pH results in extreme microbial variability (Neijssel and Tempest 1979). In addition, the compound may be unavailable to the organism if it is tightly bound to particulate matter or if the necessary transport mechanisms are inactive or absent under the experimental conditions employed.

2.1.1 Microbial Biotransformations

Drug metabolism reactions are classified as Phase I or Phase II reactions (Dutton 1978; Parke 1978; Williams 1959). A Phase I metabolic reaction is one in which a new chemical group is introduced into the molecule, especially by oxidative, hydrolytic and reductive methods. A Phase II metabolic reaction is one in which a drug or Phase I metabolite is conjugated by an enzymatic process with an endogenous molecule. Non-enzymatic Phase II reactions may occur when the substrate has functional groups which may react with endogenous molecules or solvent contaminants (Beckett *et al.* 1979). Schiff base formation by the reaction of primary amines with acetone is an excellent example. A Phase II reaction generally completes the detoxification

process and terminates the biological activity of the drug. Termination of biological activity is generally accomplished by increasing the polarity of the drug making it unavailable to the receptor sites and enhancing its rate of excretion. N-acetylation appears to be an exception to this principle as it results in decreased polarity.

Micro-organisms are known to mediate almost all types of chemical reactions under relatively mild conditions with a high degree of regio- and stereo-selectivity. These reactions are often predictable and it is possible to select a micro-organism which will mediate a particular type of reaction. There is little doubt that the various transformation mechanisms depicted by micro-organisms are not isolated curiosities but are common processes which also take place with endogenous and exogenous substances.

The widespread biochemical reactions, which micro-organisms are capable of performing, have been summarized by Kieslich (1978, 1980) and Rosazza (1980):

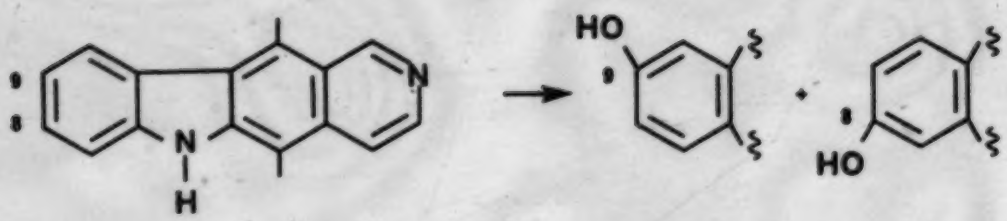
1. Oxidation: Aromatic and aliphatic hydroxylation; epoxidation; dehydrogenation; Baeyer-Villiger oxidation; decarboxylation; *ortho*, *para*-ring fission; N-oxidation; sulfoxidation; O-, N- and S-dealkylation.
2. Reduction: Ketone reduction; hydrogenation of double and triple bonds; nitro, nitroso, hydroxylamine, oxime, nitrone and N-oxide reduction.
3. Hydrolysis: Hydration of olefins; ester, amide and epoxide hydrolysis; glycoside cleavage.

4. Dehydration: Alcohols to olefins.
5. Acylation: Esterification, amide formation.
6. Conjugation: Acylation, esterification, amide formation (glycine); glucuronidation; sulfate formation; methylation; glucosidation; ribosidation; phosphorylation; acyloin condensation.
7. Isomerization and rearrangements: Dismutation, ring contraction, ring formation, *cis-trans* isomerization.

2.1.2 Microbial Oxidation Reactions

Hydroxylation is often the initial transformation route of foreign compounds although there is no clear physiological reason for this reaction (Bollag 1973). These compounds are often oxygen deficient and may need to be oxygenated to become more water soluble and biologically active. Hydroxylation reactions may occur at a saturated or unsaturated site with introduction of one or two (vicinal diol) hydroxyl groups.

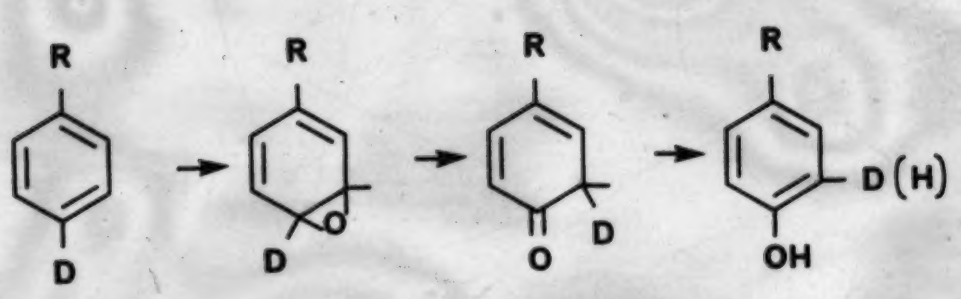
The introduction of a hydroxyl group into alicyclic or similar structures is an important microbial reaction with many examples in the transformation of steroids, alkaloids, antibiotics and other natural products (Abbott *et al*. 1977; Perlman and Peruzzotti 1970; Schoemer and Martin 1980). Hydroxylation of elipticine, an antitumour alkaloid, provides a characteristic example of this type of reaction (Scheme 3) (Rosazza 1978).



Scheme 3

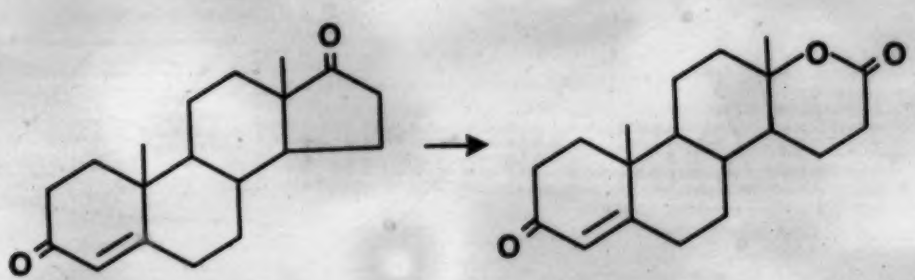
A modification of the N-substituent can change the metabolic profile of this agent. Pronounced lipophilicity of the N-substitutent promotes secondary reactions since the lipophilic compound can remain briefly within the cell after monohydroxylation. Alternatively, the compound may re-enter the cell by easily penitrating the cellular lipid barrier. A less lipophilic substrate is readily excreted after hydroxylation and would have difficulty penetrating the lipid barrier (Schubert and Siebert 1958). Increased N-substitution enhances the specificity of the direction of the reaction due to steric changes in the spacial coordination of the substrate with the enzyme (Fonken and Johnson 1972).

Boyd *et al.* (1976) have shown that fungal *para*-hydroxylation of monosubstituted aryl rings involves the migration and retention of a deuterium label via the NIH shift (Scheme 4). The hydroxylation reaction observed in *Cunninghamella bainieri* and *Aspergillus niger* is similar to that observed in plant and animal metabolism with formation of a arene 3,4-oxide intermediate (Boyd *et al.* 1976).



Scheme 4

Microbial systems can mimic known chemical reactions as seen by the oxidation of 4-androsten-3,17-dione to testololactone (a Baeyer-Villiger reaction) (Scheme 5).

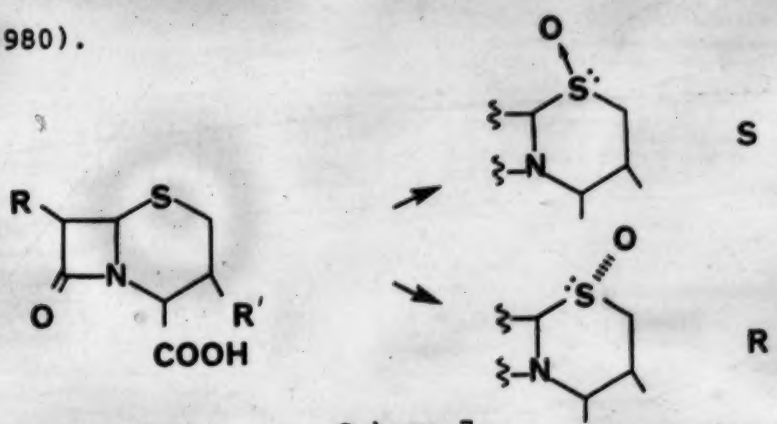


Scheme 5

In comparison with other oxidative reactions little is known about microbial N-oxidation. Hydroxamic acid formation is known in some actinomycetes (Snow 1970; Waksman 1967). The main emphasis has, however, been focused on nitro formation such as that seen in the N-oxidation of 2-amino-4-alkylimidazoles (Scheme 6) (Kieslich 1978). S-oxidation reactions have been well documented (Kieslich 1978; Smith and Rosazza 1975; Torii *et al*. 1980) and are known to be regio- and stereo-specific. A metabolically produced sulfoxide can be further oxidized to a sulfone if

Scheme 6

the reaction is left unchecked. Recent interest in S-oxidation has been observed in penicillin chemistry where several cephalosporins have been selectively oxidized by fungi to a mixture of R and S sulfoxides (Scheme 7) (Torii *et al*. 1980).

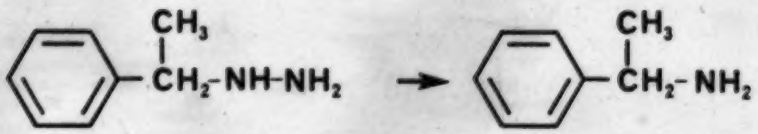


Scheme 7

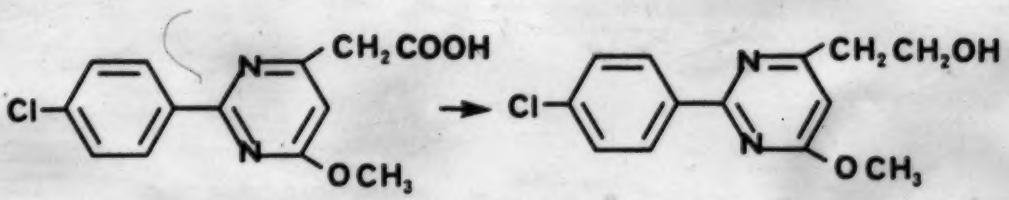
2.1.3 Microbial Reduction Reactions

Microbial reductions have been generally limited to reactions with compounds containing heterofunctions, carboxyl groups and hydrogenation of carbon-carbon double bonds (Fig. 1). Reductive reactions are less common than oxidative transformations and are generally promoted under anaerobic conditions (Savage 1977; Scheline 1973; Williams

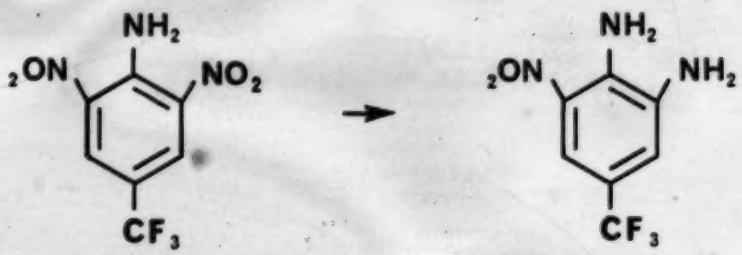
A. N-N bond cleavage (Bolton and Griffiths 1978).



B. Carboxyl group reduction (Kieslich 1978).



C. Nitro reduction (Lusby *et al.* 1980).



D. Double bond reduction (Okachi and Nara 1980).

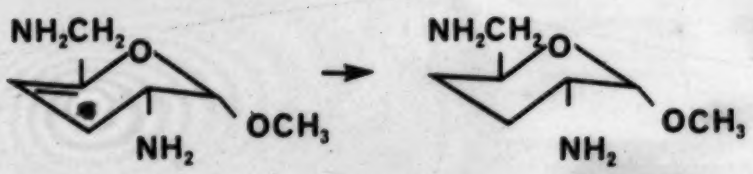
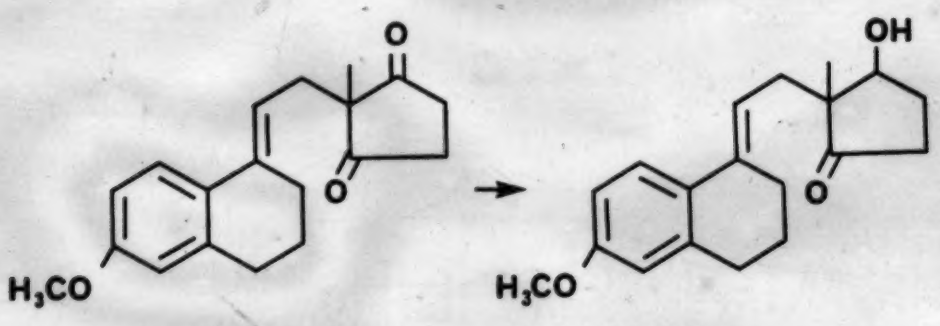


Figure 1 . Representative microbial reductions.

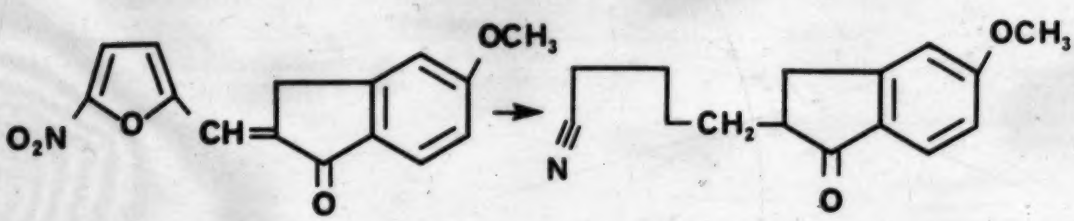
1972). However, in addition to anaerobic micro-organisms, aerobic organisms having oxido-reductases will also catalyze these reactions (Kieslich 1978).

The reduction of carboxylic acids and aldehydes are rarely observed under normal fermentation conditions. In contrast, ketones can be readily transformed as seen in the reduction of a seco-steroid (Scheme 8) (Kieslich 1978).



Scheme 8

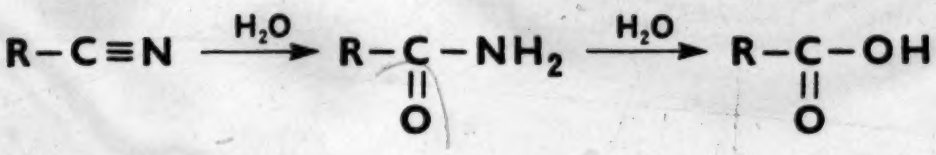
Reduction of heterofunctions is generally limited to the conversion of nitro groups to the corresponding amine. *Penicillium adamametzi* reduction of a nitrofuran to a keto-nitrile is characteristic of these reactions (Scheme 9).



Scheme 9

2.1.4 Microbial Hydrolytic Reactions

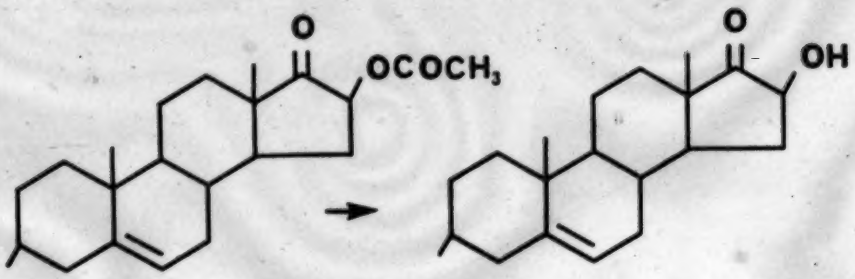
The hydrolases involved in these reactions are often extracellular and relatively stable to changes in environmental conditions (Kieslich 1978). These reactions (Fig. 2) are highly stereo-selective and will hydrate carbon-carbon double bonds, cleave carboxylic acid esters and ether bonds, and hydrolyze nitrogenous compounds such as ureas, hydrazines, semicarbazones, urethanes and lactams. Saponification of a nitrile results in the sequential formation of an amide and the corresponding acid (Scheme 10) (Harper 1977).



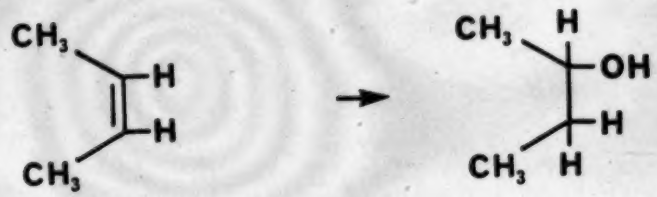
Scheme 10

It is evident from the studies of Udenfriend and his associates in 1954 that nonspecific hydrolysis can result chemically (non-enzymatically) *in vitro* under physiological conditions in a manner closely analogous to the *in vivo* transformation. Hydrolytic reactions may be enzymatically induced but the reaction may also be chemically mediated by exposing the compound to alkaline or acidic conditions. This makes it difficult to determine whether hydrolysis is a result of a primary biological or a secondary reaction

A. Hydrolysis of carboxylic acid esters.



B. Hydration of carbon-carbon double bond.



C. Hydrolysis of ethers.

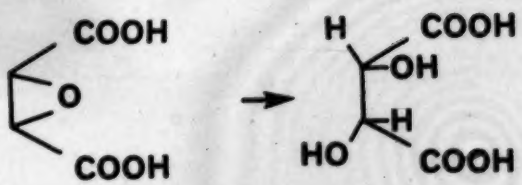
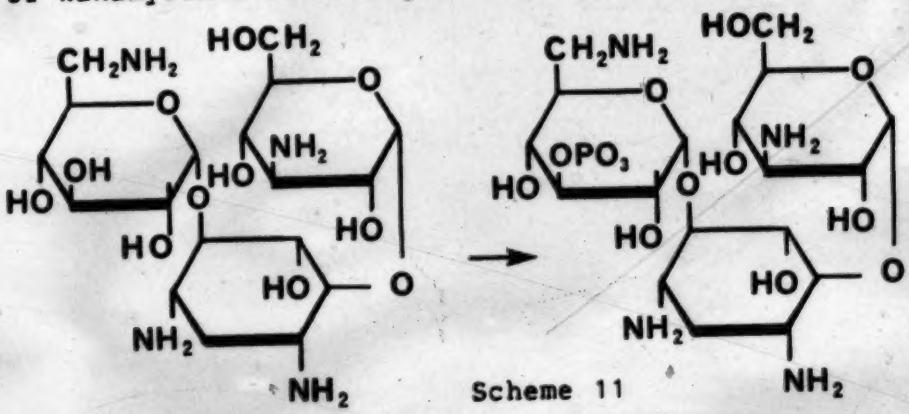


Figure 2 . Examples of microbial hydrolytic reactions.

resulting from biological changes in pH, inducing a chemical reaction. Occasionally the only method available to differentiate between biological or chemical reactions is their response to temperature changes. The rate of any chemical or biological is increased when the temperature is raised. Obviously there are temperature optima for both the biological and chemical reactions, the biological reaction having the lower maximum due to thermal denaturation of proteins and enzymes.

2.1.5 Microbial Conjugative Reactions

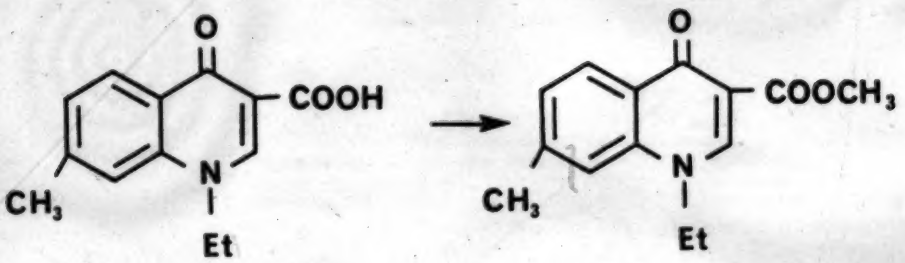
Microbial anabolic reactions are based on enzymatic transfer of activated acetate, phosphate, methyl group or glucose to a suitable amino or hydroxyl function (Fig. 3). Interesting microbial parallels to a number of conjugative reactions have been observed, including the phosphorylation of kanamycin A to kanamycin 3-phosphate (Scheme 11)



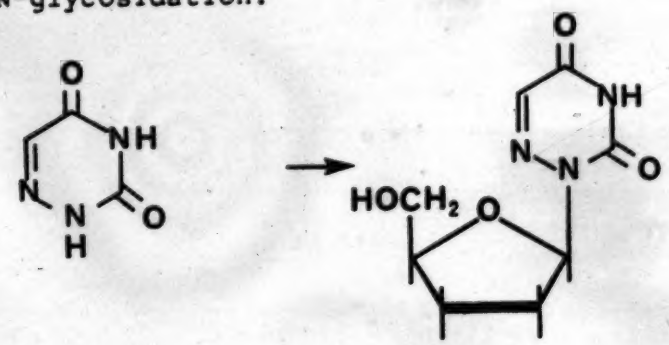
Scheme 11

by *Staphylococcus aureus* and streptomycin adenylation by *Escherichia coli* (Sebek and Perlman 1971).

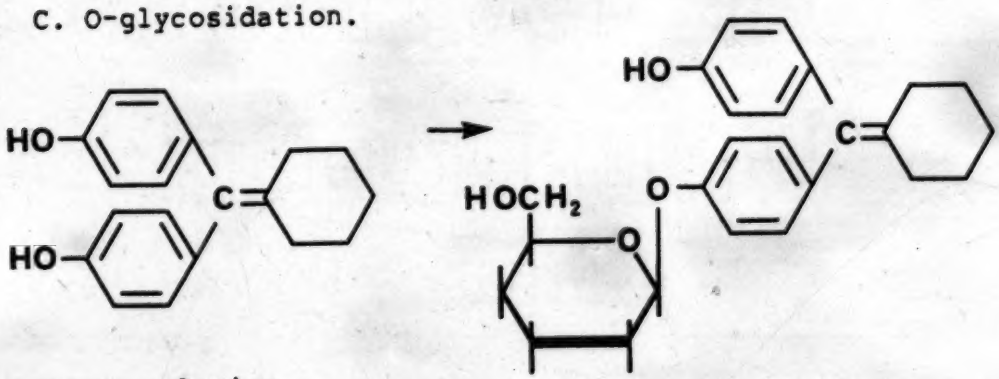
A. O-methylation.



B. N-glycosidation.



C. O-glycosidation.



D. N-acylation.

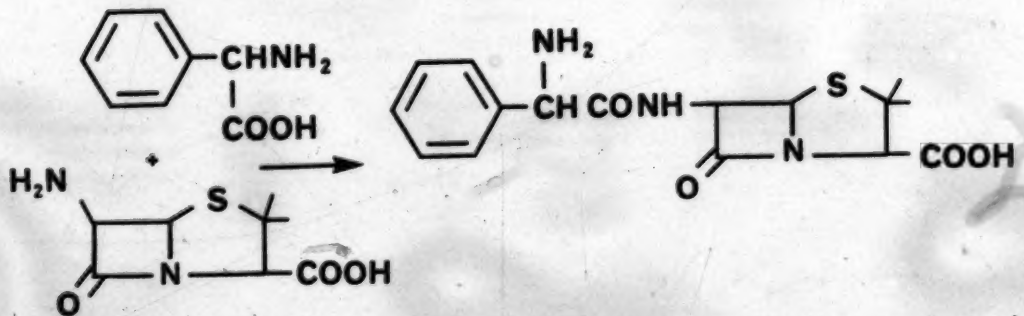


Figure 3 . Representative examples of microbial conjugations.

These typical reactions show that a wide variety of substrates are amenable to microbial transformation for the purpose of microbial modelling or metabolite production.

2.1.6 Microbial Models

The appropriateness of microbial models of mammalian metabolism have been verified by several groups (Cerniglia and Gibson 1979; Perlman and Peruzzotti 1970; Smith and Rosazza 1975). The metabolic profiles of ellipticine, danazol, spironolactone analogues, papaverine, miscellaneous antiinflammatory agents and prostaglandin analogues in both microbial and mammalian systems are remarkably similar (Rosazza 1980).

The utility of microbial models may be illustrated by examination of two of the numerous examples known: benzo[a]pyrene and acronycine. Benzo[a]pyrene was oxidized by *Cunninghamella elegans* to several metabolic products (Fig. 4) (Cerniglia and Gibson 1979). Experiments showed that over a 96 h period 18% of the substrate was transformed. The products were mainly recovered as glucuronide or sulfate conjugates. The metabolic pattern observed in this microbial system was remarkably similar to that found in higher organisms.

Acronycine is highly active against a variety of tumour test systems (Rosazza and Smith 1979). In mammalian systems this compound was extensively metabolized at the 9- and 11-positions (Fig. 5). In addition, one of the *gem*-dimethyl

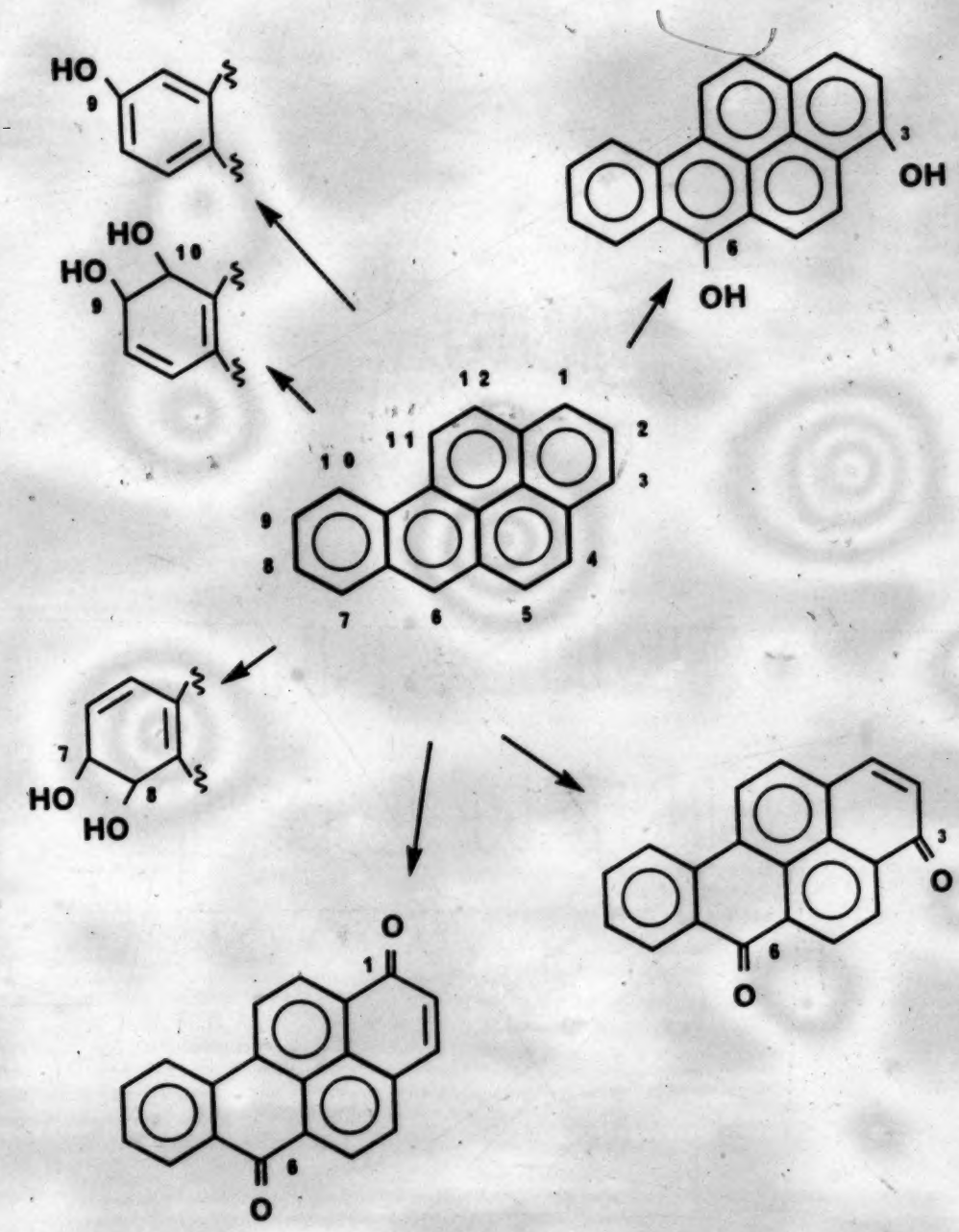


Figure 4 . Microbial and mammalian biotransformation of benzo[a]pyrene (Cerniglia and Gibson 1979).

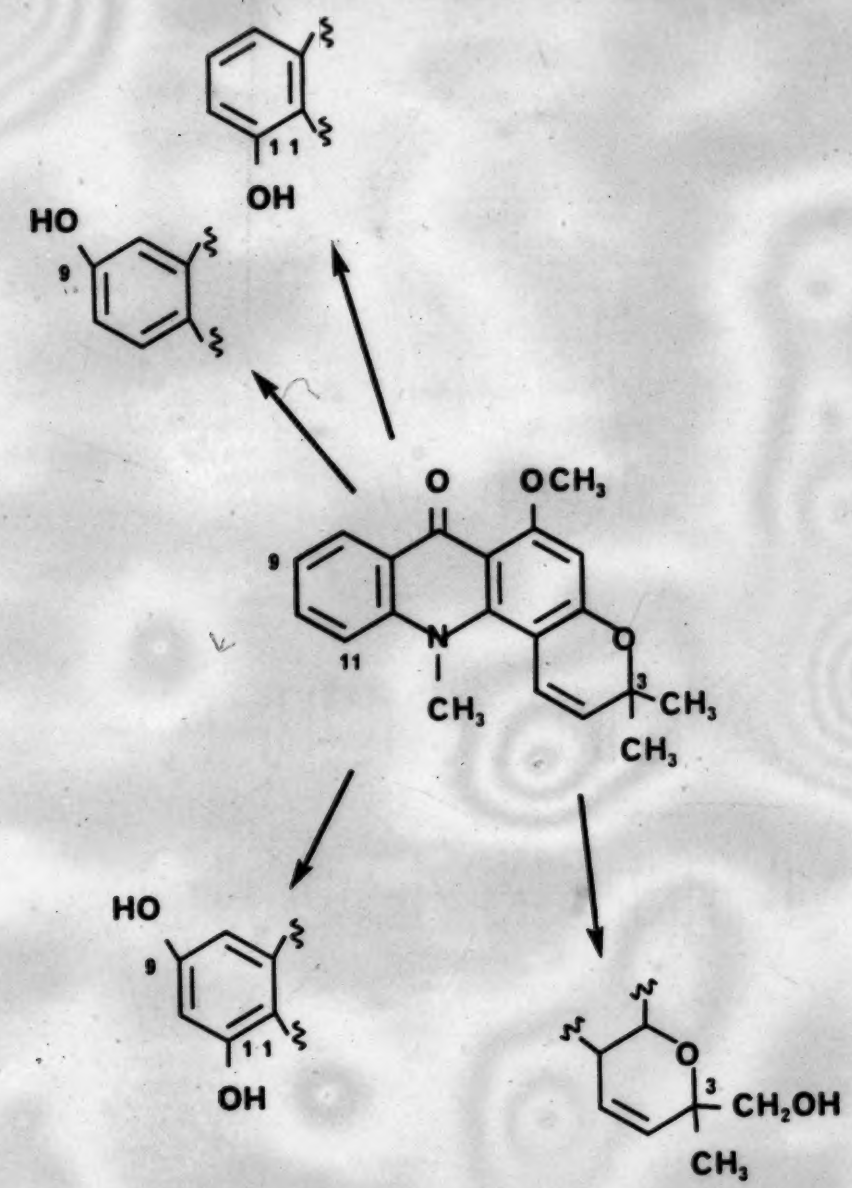


Figure 5 . Mammalian and microbial metabolic profile of acronycine (Rosazza and Smith 1979).

groups at position 3 was hydroxylated in man, rat, dog and mouse. Minor quantities of the 6-demethylated and dihydroxy metabolites were also recovered. *Cunninghamella echinulata* gave a 30% yield of the 9-hydroxyacronycine and 3-hydroxy-acronycine was recovered from *Aspergillus alleaceus* incubation broths (Rosazza and Smith 1979). All of the transformation products were obtained in sufficient quantities for complete chemical and biological testing.

Advantages to the employment of microbial models include the production of gram quantities of metabolites through routine scale up procedures. This is useful as many of the mammalian metabolites are formed in minute quantities. For example, danazol is metabolized in mammalian systems to four products in trace amounts. These four metabolites have been synthesized in 20-70 g amounts by several micro-organisms (Rosazza 1980).

The feasibility of using microbial systems as models of mammalian metabolism is supported by the similarities observed with microbial and mammalian drug metabolizing enzymes (Schunck *et al.* 1978; Wiseman 1980). They have essentially the same components and similar requirements for NADPH or NADH as cofactors, nonheme iron protein, a flavo-protein and cytochrome P-450 monooxygenase (Cerniglia and Gibson 1978; Ferris *et al.* 1973; Ferris *et al.* 1976; Rosazza 1980; Schunck *et al.* 1978).

2.2 Micro-organism Selection

The selection of an organism and the methods used for the maintenance of a culture of the organism are among the most important decisions that have to be made in biotransformation studies. Actinomycetes, bacteria and fungi differ widely in their response to xenobiotics (Abbot and Gledhill 1971; Beukers *et al.* 1972; Bollag 1973; Sebek 1974; Smith and Rosazza 1975). Perlman and Kikuchi (1977) have listed the general attributes required by these micro-organisms:

1. The strain must be genetically stable;
2. The strain should be readily maintainable for reasonable periods of time;
3. The strain must readily produce vegetative cells, spores or other reproductive units;
4. The strain should grow vigorously and rapidly in the medium used;
5. The strain should be free from other micro-organisms, including bacteriophages;
6. The strain should be able to protect itself from contamination; and
7. The strain should be easily modified by certain chemicals or a group of mutagenic agents.

Microbial life is a series of controlled chemical reactions and the velocity of these reactions can be expressed as a kinetic equation in which the velocity is a function of the reactant concentration. The rate of biotransformation is directly proportional to the substrate

concentration. It is known, however, that some biological reactions are not observed when the substrate concentration is below a threshold value (Alexander 1980). The overall yield of a biological reaction is dependent on four factors: substrate concentration, the cell biomass, the specific enzymatic rate and the duration of the reaction.

The distinction between biologically synthetic or destructive forces in drug metabolism studies are minute, but these forces can be controlled and accelerated to advantage through an understanding of the mechanisms involved. To increase the production of a metabolite, many organisms can be selectively improved by standard techniques:

1. Selection alone: the best technique is to test many strains for approximate production rather than test a few accurately. Enrichment culturing is a common selection method.
2. Mutation, followed by selection: the organism is treated with ionizing or UV radiation, chemical mutagens, nitrous acids, and alkylating agents.
3. Recombination, followed by selection: provides a new capability of altering organisms but it is difficult to use with fungi (Johnston 1975).

If several enzymes are involved in the biotransformation process (Nebert 1979), the reaction can be controlled by the addition of selective modifying agents or through structure manipulation (Kogut 1980; Martin 1978).

Classically, modification can be regulated by using specific enzyme inhibitors (Schunck *et al*. 1978) or by altering the source of either carbon or nitrogen (Drew and Demain 1977; Martin and Demain 1980; Rose 1979).

The principle goal of all micro-organisms is survival. To achieve this end, growth and reproduction must follow a highly ordered and intricate series of reactions. Every environment exerts differing challenges and the organism will only survive if the environment does not select against it. Many micro-organisms will adapt to the environment, possibly with the loss of a phenotype, a phenomenon referred to as strain degeneration. To the organism this is strain improvement.

2.2.1 Incubation Conditions

Two techniques are commonly used to study microbial biotransformation, co-metabolism (Horvath 1972; Leuenberger 1978; Perry 1979; Smith and Rosazza 1975) and culture enrichment (Schlegel and Jannasch 1967; Veldkamp 1970). Co-metabolism is the process in which a micro-organism metabolizes a non-growth substrate in the presence of a utilizable carbon and nitrogen source. Culture enrichment is where several organisms are incubated in a defined minimal medium with the substrate to be transformed as the sole source of carbon and or nitrogen in an attempt to obtain one or more organisms capable of metabolizing the substrate completely. Co-metabolism has been employed as the major

technique for the biochemical study of microbial metabolism. Because co-metabolism involves relatively few transformation steps, the substrate used in the biotransformation study will not be metabolized to inorganic carbon and products will accumulate in the culture fluid. For example, co-metabolism was used in the *Nocardia rubra* biotransformation of steroids into equilin (Horvath 1972).

The use of enrichment culture is a less promising technique for biotransformation studies as the substrate is generally transformed into products of intermediary metabolism. This was observed in *Pseudomonas putida* catabolism of (-)-ephedrine in enrichment culture to methylamine, formaldehyde, methylbenzoylcarbinol, benzoic acid, pyrocatechol and *cis,cis*-muconic acid (Klamann and Lingens 1980; Klamann *et al.* 1980).

The choice of a medium for co-metabolism studies is based primarily on the nature of the organism and the direction in which the reaction is to proceed, namely catabolic or anabolic (Beech and Davenport 1971; Booth 1971; Bridson and Brecker 1970). Utilization of medium nutrients and the accumulation of metabolic products will influence the pH of the incubation broth (Neijssel and Tempest 1979). Temperature effects (Farrell and Rose 1967) and medium pH will affect growth rate, morphology and production of metabolites. Biological buffers can be used to ensure optimal pH conditions but may occasionally produce unwanted side reactions (Ferguson *et al.* 1980; Neijssel and Tempest

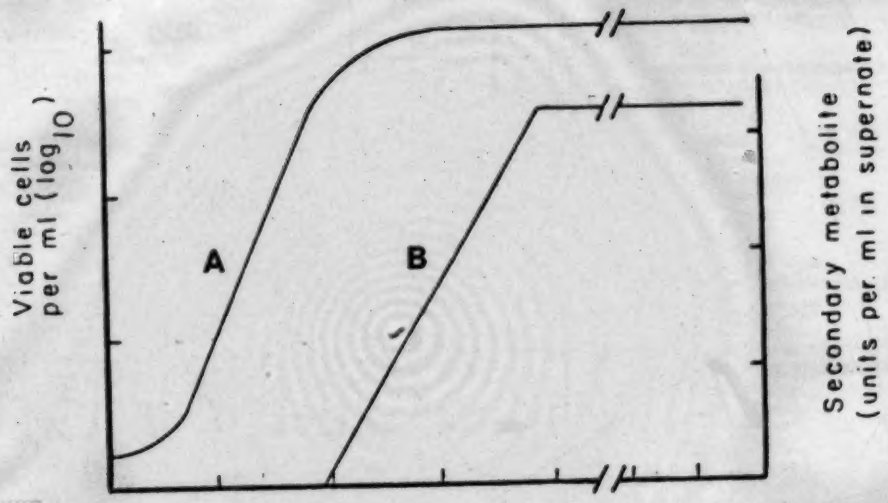
1979; Weinberg 1970).

2.2.2 Microbial Growth Factors

Many metabolites are produced or modified during different times of microbial growth. In an unrestricted system where the organism is grown under optimal nutrient and environmental conditions, growth is an auto-catalytic process (Fig. 6) (Righelato 1975). The unrestricted growth phase is generally short in duration and is followed by a phase of decelerating growth, the restricted phase. This term refers to any growth condition where the growth rate is not maximal as a result of a limiting environment, often resulting from microbial modification of the biosphere.

The enzymes of unrestricted (primary) metabolism are often highly specific, whereas in restricted (secondary) metabolism this degree of specificity is not always encountered (Bu Lock 1975). On this basis, it is possible to manipulate the metabolic profile observed. The concept of unrestricted (primary) and restricted (secondary) metabolism is not definitive as some supposedly restricted reactions have been observed during the active (unrestricted) growth phase (Drew and Demain 1977). Demain suggested that the only valid definition of a secondary metabolite is that it is a compound which is not required for growth of the organism that produced it.

Surface growth of actinomycetes and fungi on liquid or solid media is heterogenous, composed of aerial, surface and



Terms used to differentiate microbial phases of growth.

Primary	Secondary
Unrestricted	Restricted
Trophophase	Iodophase
Growth	Production

Figure 6 . Schematic representation of microbial growth (A) and production of a biotransformation product (B).

subsurface hyphae, each in a different physiological state. In shaken (submerged) liquid culture growth tends to be homogenous. The culture can become heterogenous if the apparent viscosity of the medium restricts adequate agitation of the organism. This transformation from homogenous to heterogenous growth can stop or influence the biotransformation by the organism.

Pellet growth has a lower apparent broth viscosity than an equal amount of filamentous cells (van Suijdam *et al.* 1980). The advantage of pelleted cells is that the cells have better mixing and mass transfer properties than free cells and it is easier to analyze for metabolite production. The main disadvantage of pelleted cells is that as the pellet size increases nutrients cannot diffuse inwards at a rate fast enough to maintain unrestricted growth of the whole pellet mass (Righelato 1975). Extremely dense pellets (sclerotia) are produced in response to adverse environmental conditions. The cells are in a resting phase and are metabolically viable (Smith 1975).

The importance of the inoculum size and quality has long been recognized to affect both growth pattern and efficiency of growth. Morphological observations made relative to the metabolic capabilities of the organism are not reliable and are a poor method of assessing inoculum quality. Variations in culture morphology can be achieved by seeding the medium with different amounts of cells or spores and altering the environmental conditions (Grootwassink and

Gaucher 1980).

3. Materials and Methods

3.1 Chemicals and Reagents

Materials obtained from commercial sources were: *p*-chloropropiophenone, Aldrich; molecular sieves, anthracene, British Drug Houses; lithium aluminum hydride (LAH), sodium borohydride (SBH), Baker Chemicals; acetoin (3-hydroxy-2-butanone), chloroethanol, 4-hydroxy-2-butanone, *o*-phenylenediamine, Eastman Chemicals; CO, semicarbazide HCl, Matheson Coleman and Bell; Surfasil, BSTFA, BSA, Pierce Chemicals; *p*-chlorophenylethylamine, diacetyl (2,3-butanedione), glutaraldehyde (grade II), 2,3-dimethylquinoxaline, dimethyl dichlorosilane, Sigma; and SKF-525A, Smith Kline and French. Pargyline-HCl, pheniprazine, phenelzine, tranylcypromine, fluphenazine and (-)-deprenyl-HCl were gifts from Dr. G.B. Baker, Department of Psychiatry, University of Alberta. The N-1-formylethyl derivative of PLZ was a gift from Dr. T. Danielson. N-methylamphetamine was purchased from Abbott Labotatories Ltd. and benzylamine was obtained from Eastman Kodak. Solvents and chemicals were of reagent grade and used without further purification unless stated otherwise.

3.2 Culture Maintenance

Three classes of micro-organisms were used in this study: fungi (Table 2), aerobic bacteria, and actinomycetes (Table 3), and clinical isolates (anaerobes) (Table 4). With the exception of clinical isolates and cultures obtained from the University of Alberta Mold and Culture Herbarium (UAMH), all cultures were initially grown on solid media to assess for strain purity and ensure that the culture was free of contaminating micro-organisms and where relevant, bacteriophages.

Bacteria, yeasts and actinomycetes used in this study were preserved by lyophilization and running cultures were maintained on nutrient agar slants, *M. smegmatis* was maintained in nutrient broth (Lapage *et al*. 1970; Onions 1971; Perlman and Kikuchi 1977). Fungi were kept on oatmeal agar maintenance slopes to ensure limited metabolic activity. Alternatively, fungal spores were suspended in sterile distilled water. All cultures were stored in a refrigerator at 4°C. Running cultures were periodically transfered to fresh maintenance media to retain culture vigor and ensure culture purity.

3.3 Incubation Conditions

A simple two stage incubation procedure was used for all micro-organisms tested in this study. Cells and spores from fresh maintenance cultures were suspended in sterile

Table 2 . Fungal cultures examined in these studies.

Organism	Source
Alternaria tenuis NEES	UAMH 1793
Aspergillus alliaceus	UAMH 2976
Aspergillus niger	ATCC 9142
Aspergillus ochraceus	ATCC 1008
Chrysosoporium pruinosum (state of Phanaerochaete)	UAMH 3642
Cunninghamella echinulata	ATCC 3655
Cunninghamella echinulata	ATCC 9244
Curvularia inaequalis	UAMH 1406
Cylindrocarpon tonkinense	UAMH 1952
Gliocadium roseum	UAMH 3477
Gliocadium viride	UAMH 1365
Microsporum cookei	UAMH 2937
Microsporum gypseum	UAMH 1615
Mucor pusillus	UAMH 2170
Penicillium notatum	UAMH 485
Rhizopus nigricans	UAMH 1947
Saccharomyces cerevisiae HANSEN 1883	UAMH 199
Schizosaccharomyces octosporus	UAMH 202
Trichophyton terrestre (state of Arthroderma quadrifidumt)	UAMH 1848
Trichothecium roseum	UAMH 3015
Trichurus spiralis	UAMH 3585

Table 3 . Aerobic bacteria and actinomycetes screened for their ability to N-oxygenate N-(*n*-propyl)amphetamine.

Organism	Source	
Alcaligenes faecalis	NCIB	8156
Escherichia coli	ATCC	8739
Escherichia coli	NCIB	8879
Klebsiella sp.	NCIB	9111
Mycobacterium smegmatis	ATCC	14468
Pseudomonas sp.		
Pseudomonas sp.		
Pseudomonas oleovorans	NCIB	6576
Pseudomonas sp.	NCIB	8194
Pseudomonas aeruginosa	NCIB	8295
Pseudomonas sp.	NCIB	8858
Salmonella typhimurium	SGSC	LT2
Salmonella typhimurium	SGSC	SA35
Serratia marcescens	ATCC	13880
Staphylococcus aureus	BX	1633-2
Staphylococcus epidermidis		
Streptomyces platensis	ATCC	13865
Streptomyces platensis	ATCC	13886
Streptomyces sp.	UAMH	2950

Table 4 . Clinical isolates obtained from the University of Alberta Hospital which were screened for their ability to effect anaerobic biotransformation of N-methylamphetamine.

Bacteroides fragilis	16758
	16804
	16806
	16813
	17062
	17069
Clostridium	17086
	16876
	CU 1756
Clostridium perfringes	
Clostridium ramosum	
Enterobacter cloacae	
Lactobacillus	16976
	17265
	17060
	17087
Streptococcus faecalis	

medium. Surface growth or spores were removed from the maintenance cultures by a nichrome inoculating loop and added to the medium aseptically. Alternatively, cells kept in liquid medium were added to fresh media by a pipet. These first stage cultures were incubated until the cells approached the maximal culture density and growth rate. Bacteria, yeasts and actinomycetes were incubated overnight and the actively growing cultures were used to inoculate stage II flasks containing the same medium. Fungi were incubated for 72 h before the biomass and growth rate were suitable. Fungal cultures were then homogenized in a Waring blender in an attempt to standardize the inoculum by having a homogenous suspension of cells. The homogenate was added as a 10% inoculum to stage II flasks containing the same medium.

The diverse growth requirements of the micro-organisms necessitated the use of various media. Bacteria, yeasts and actinomycetes were routinely grown in Difco nutrient broth, supplemented with dextrose, 16g/L. Anaerobic cultures were grown in Baltimore Biologicals Ltd. (BBL) Thioglycolate broth. Trypticase soy broth (BBL) supplemented with dextrose 16g/L was the major medium for fungal studies; additional media were screened for their ability to support and enhance biotransformation (Table 5). Microbial transformation experiments were conducted in either 125 mL Erlenmeyer flasks containing 25 mL of medium (aerobic) or in 15 mm X 150 mm test tubes containing 10 mL of medium (anaerobic).

Table 5 . Media used in this study, quantities given in g/L.

A. Modified trypticase soy broth (pH 7.2, KOH)			
trypticase soy broth	30	KH_2PO_4	5
yeast extract	5	dextrose	40
B. Defined minimal medium (pH 7.4, KOH)			
$NaNO_3$	2	KH_2PO_4	1
$MgSO_4.7H_2O$	0.5	$CaCl_2$	0.1
$FeSO_4$	0.1	KCl	0.1
dextrose	40		
C. Soya bean medium (pH 7.0, KOH)			
yeast extract	5	NaCl	5
soya bean meal	5	K_2HPO_4	5
dextrose	40		
D. Corn steep liquor medium (pH 6.8)			
trypticase soy broth	30	corn steep liquor	5

Fungal cultures were incubated at 28 °C on a New Brunswick Scientific Co. Model G-10 gyrotory shaker, equipped with 45° angle brackets, operated at 250 rpm. However, in some cases, stage II flasks were incubated in a stationary mode, allowing a surface mycelial mat to form. Substrates were dissolved in water and 1 mL aliquots were added aseptically to each 48 h stage II flask by filtration through a 0.22u filter disc in a Sweeney filter (Millipore Corp.).

Anaerobic metabolic studies were performed with the Gaspak system (BBL) consisting of anaerobic jar, catalyst, hydrogen and carbon dioxide generator and anaerobic detector. Substrates in 0.2 mL of degassed water, were added aseptically at the initiation of stage II incubation.

Bacterial, yeast and actinomycete cultures were incubated at 37°C in a New Brunswick Scientific Co. Model G-25 gyrotory shaker incubator, equipped with 45° angle brackets, operated at 250 rpm. Substrates, in 1 mL of water, were added aseptically after 6 h of stage II incubation as described previously.

Where possible a single lot of all chemicals and media was used throughout this study. It was necessary though to use several lots of nutrient broth, dextrose and trypticase soy broth. Media and dextrose solutions were autoclaved at 22 psi, 262°F (122°C) for 15 min in a Amsco general purpose autoclave. In the last stage of this study, all incubation flasks were treated with Surfasil siliconizing fluid to

maintain submerged culture homogeneity by preventing surface growth on the flask wall. The solution was applied after every second incubation.

Preparation of media was standardized by using the same balance, weights and glassware. Double glass distilled water from a Corning Ag11 still (setting 300K) was used routinely for all experiments. Glassware was washed with Alconox detergent and rinsed in a Miele G19 automatic dishwasher and finally rinsed with deionized water.

Culture controls were prepared by incubating the micro-organism in the absence of substrate. Samples of each culture were plated on Difco Nutrient and Sabouraud dextrose agar. Substrate controls were prepared by incubating substrates in sterile medium.

3.4 Analysis

The diverse nature and reactivity of substrates and their metabolites meant that one analytical procedure was inadequate to examine the incubation broths for all possible metabolites.

3.4.1 Extraction Procedures

All incubation broths were analyzed by withdrawing 1-3 mL aliquots at various times up to 168 h. With some substrates three different sampling methods were necessary to assay the incubation broths for all possible products. In

such cases each aliquot was divided into three equal 1 mL portions. Method A: A 1 mL sample was made alkaline (solid potassium carbonate) and extracted three times with five volumes of a diethyl ether-methylene chloride mixture (55:45 v/v). The solvents were freshly distilled prior to use. The extracts were combined and concentrated at 50°C to approximately 50 uL. Method B: A 1 mL sample was made acidic (concentrated HCl) and extracted as outlined above. The combined extracts were evaporated to dryness and derivatized with 50 uL of chloroethanol/HCl at 50°C for 15 minutes. The reaction mixture was reduced to dryness under a stream of nitrogen and the residue was redissolved in the diethyl ether-methylene chloride mixture to a final volume of 50 uL. Method C: Since phenols have a poor extraction efficiency under alkaline conditions, it was necessary to derivatize these compounds prior to extraction. The aqueous sample was basified with either potassium carbonate (pH 9.5) or sodium bicarbonate (pH 8.5) and mixed (vortexed) rapidly with 100 uL of acetic anhydride in 5 mL ether-methylene chloride for 2 min. The solvent layer was removed and the extraction was repeated once. The organic layers were combined and reduced to dryness at 55°C. The residue was redissolved in solvent and analyzed.

3.4.2 Acylation

In addition to Method C (section 3.4.1), three alternative acylation procedures were routinely used, depending on the sample and reaction conditions. Method D: A 10 uL aliquot of the extract (Method A, section 3.4.1) was reacted with 50 uL of either acetic anhydride or trifluoroacetic anhydride. The solution was allowed to stand for 15 min at room temperature and then reduced to dryness under a stream of nitrogen. The residue was redissolved in ether-methylene chloride and analyzed. Method E: Anhydrous acylation was performed by adding 1 mg of authentic standard and 5 mg sodium bicarbonate to a solution of 200 uL of ether and 50 uL of acetic anhydride or trifluoroacetic anhydride. The reaction mixure was vigorously vortexed for 5 min and the solution was concentrated and analyzed as above. Method F: On-column acetylation of the sample with acetic anhydride. An 1 uL aliquot of this reagent was injected into the gas chromatograph with 1 uL of the sample. The reaction was spontaneous.

To remove excess reagent in TFAA reaction mixtures (Methods D and E), the mixtures were washed with a saturated solution of sodium borate prior to reducing the sample to about 50 uL.

Fluphenazine and its metabolites were extracted from aqueous media (Method A) and the extract was reduced to dryness. The sample was dissolved in tetrahydrofuran (200 uL), triethylamine (100 uL) and reacted with acetic

anhydride (100 uL) for 5 min at room temperature; the sample was reduced to dryness and dissolved in methanol for HPLC analysis.

3.4.3 Silylation

Silylation of most hydroxyl compounds was generally complete within 15 min. The N,O-bis(trimethylsilyl)acetamide (BSA) derivative of N-hydroxy-N-propargylbenzylamine was prepared by adding 50 uL of BSA reagent to a portion of the original extract which had been reduced to dryness under nitrogen. The solution was maintained at 55°C for 1 h, reduced to dryness under nitrogen, then redissolved in a small amount of ether-methylene chloride. Derivatization with N,O-bis-(trimethylsilyl)trifluoroacetamide was conducted in a manner similar to that reported above except the residue of the alkaline extract was redissolved in 200 uL of acetonitrile and 50 uL of BSTFA.

3.4.4 Chemical Reductions

SBH reduction products were formed by reducing a 20 uL portion of the original extract, which has been previously dried with molecular sieves, and evaporated to dryness. The residue was redissolved in 50 uL absolute ethanol and reacted with 1 mg SBH for 0.5 h. The reaction was stopped by the addition of 100 uL of 1% HCl. The mixture was basified and the sample was extracted with 50 uL of solvent and analyzed by GLC. Alternatively, reduction products were

formed by reacting a 20 uL aliquot of the original sample with 1mg LAH in anhydrous ether for 0.5 h. The reaction was stopped by addition of 200 uL of 1% HCl, basified, extracted with 50 uL solvent and analyzed by GLC.

3.4.5 Gas-liquid Chromatography

Gas-liquid chromatographic (GLC) analysis was performed on a Hewlett-Packard Model 5700A gas chromatograph equipped with a flame ionization detector. GLC spectra were recorded on a Hewlett-Packard Model 3380A integrator. Three different columns were used for analysis: Column A, 5% OV-101 on Chromosorb 750 (McReynolds constant, 217) (80/100 mesh) in a glass column, 4mm internal diameter and 1.68m in length; Column B, 1% Carbowax 20M (McReynolds constant, 2308) on Chromosorb 750 (80/100 mesh) in a glass column, 4mm internal diameter and 1.26 m in length; Column C, 1% OV-17 (McReynolds constant, 884) on Chromosorb 750 (80/100 mesh) in a glass column, 4mm internal diameter and 0.84m in length. Constant operating conditions were: carrier gas (He), 60 mL/min; Hydrogen gas, 40 mL/min; air, 240 mL/min; injector port and detector temperatures, 250°C. Empty glass columns were treated with 5% dimethyl dichlorosilane in toluene to deactivate active sites on the column (Coutts and Jones 1980). *p*-Chloropropiophenone was used as the internal standard.

3.4.6 Gas-liquid Chromatography/Mass Spectrometry

Electron impact mass spectrometric (MS) analysis was carried out on a Hewlett-Packard Model 5710A gas chromatograph/Model 5918A mass spectrometer/Model 5934A data system. The injection port and transfer line temperature was 250°C. The operating conditions for the mass spectometer were: electron energy, 70 ev (electron volt, 1eV=0.16021 aJ); emission ion current, 35 mA; source temperature, 180°C; separator temperature, same as the oven temperature.

Chemical ionization mass spectrometry was performed on a Hewlett-Packard model 5985A spectrometer with dual electron impact-chemical ionization source. Methane was used as the carrier and reactant gas. Source pressure was 0.8 Torr(1 Torr = 133.322Pa) and electron energy was 175 eV.

The columns used for GLC were also used for combined GLC-MS, with He, 60 mL/min, as the carrier gas.

3.4.7 High Performance Liquid Chromatography

HPLC was performed by Dr. Locock on a Water Associates Liquid Chromatograph equipped with a model 440 absorbance detector and connected to a electrochemical detector. Separations were performed on a C18-microbondapak column, 4 mm by 30 cm . The solvent was a 5 mM pentane sulfonic acid solution in 40% methanol containing PIC-B5 (Water Associates). The flow rate was 1 mL/min.

HPLC retention times for authentic standards were determined to be: *p*-hydroxyamphetamine, 4.25; amphetamine,

6.0; N-hydroxyamphetamine, 7.5; 1-phenyl-2-propanone oxime (syn), 7.5; 1-phenyl-2-propanone oxime (anti), 10.5; 1-phenyl-2-propanone, 8.35; 1-phenyl-2-propanol, 10.1; N-acetylamphetamine, 9.1; fluphenazine, 24.4; and fluphenazine sulfoxide, 8.1 min.

3.4.8 Thin-layer Chromatography

Chromatograms of extracts and substrates were run on silica gel G (Eastman) or cellulose (Merck) plastic plates. Precoated plates containing a material which fluoresces under UV light (254 nm) were used occasionally. Detection occurred when compounds able to absorb energy of this wavelength quenched the fluorescence leaving a darkened spot. The solvent systems used were: A, chloroform-methanol (9:1 v/v); B, methanol-acetone (1:1 v/v); C, ether; D, ethyl acetate; and E, ethyl acetate-benzene (1:1 v/v). Spots were detected by iodine vapour, ammoniacal siver nitrate (Tollen's reagent), short wave UV light (254 nm), long wave UV light (280 nm) or Dragendorff reagent (Coutts and Beckett 1977). Tollen's reagent (5% silver nitrate in 0.88M ammonium hydroxide) was prepared daily. A TLC spot could be eluted from the solid support and further analyzed by GLC.

A portion of the solvent extract was reduced to dryness in a culture tube and allowed to react with Tollen's reagent for 0.5 h or until the dark colour has fully developed. The mixture washed with 1% HCl and then 1% ammonium hydroxide. The resulting alkaline mixture was then extracted with

ether-methylene chloride and analyzed by GLC.

3.5 Cell Immobilization

3.5.1 Bead Preparation

Cultures of *C. echinulata* grown in trypticase soy broth and dextrose were harvested at either 72 h of stage I growth or 48 h of stage II growth by vacuum filtration. The filtered mycelia were resuspended in 25 mL of sterile distilled water and homogenized in a Waring blender until the homogenate could readily pass through a 20 gauge needle. The cellular suspension was warmed to 55°C and added to a sterile solution (100 mL) of 2.2% carrageenan (Sigma, type I) in the initial studies on fungal immobilization. Later studies were performed using a 2% final concentration of NJAL 724 carrageenan obtained from the FMC Corporation with the compliments of Mr. J.T. Whitt, Marine Colloids Division. In the latter studies the resulting suspension was kept at 45°C until the beads were formed by extrusion of this suspension through a 20 gauge needle, using a 50 mL glass syringe, into the gelling solution.

The gelling solution (200 mL) consisted of a mixture of 2% potassium chloride and 0.015% calcium chloride dihydrate and was stirred gently with a magnetic stirring bar during the bead extrusion process. Routinely, no more than 90 mL of the carrageenan-fungal suspension was added to each

container of gelling solution. The bead shape was influenced by both the height from which the bead was extruded into the gelling solution and the speed at which this solution was mixed with a magnetic stirrer. The spherical nature of the beads decreased with increasing height and mixing speed. The beads generally obtained were oval. Spherical beads were formed only when the gelling solution was stationary but such beads momentarily floated just below the liquid-air interface and coalesced during the first few moments. This problem was overcome by using a larger container for the gelling solution but sepsis could not be maintained.

The beads prepared from Sigma carrageenan were hardened for 1 h at 4°C by adding 50 uL glutaraldehyde to the gelling solution containing the beads. The solution was filtered and washed twice with two volumes of gelling solution to remove remaining traces of glutaraldehyde. Alternatively, the beads were soaked for 1.5-2 h in the gelling solution, washed with sterile gelling solution and then added to the reactor. Beads prepared from FMC carrageenan were not treated with glutaraldehyde after formation. The beads were used in biotransformation studies after washing with saline to remove excess KCl. Aseptic technique was followed at all stages of bead extrusion and reactor filling.

3.5.2 Fluidized Bed Reactors

Two types of reactor were commonly employed for these immobilization studies, a batch reactor and a fluidized bed tower reactor. The basic batch reactor was comprised of 125 mL Erlenmeyer flasks used routinely for biotransformation studies. In this system, immobilized cells were added to 25 mL of either trypticase soy broth or phosphate buffer, 0.05 M, pH 7.2, and incubated with shaking as outlined in the chapter on incubation conditions for fungal cultures (section 3.3). In distilled water at either 27° or 37°C the beads dissolved. Bead integrity was maintained with addition of 1% KCl or 0.05M potassium phosphate to the incubation medium.

Carrageenan beads containing *C. echinulata* were intact after 21 days of incubation at 27°C at 200 rpm in a batch reactor with trypticase soy medium. The carrageenan beads were removed from this reactor, filtered, washed with distilled water and allowed to stand in distilled water until the beads dissolved. The free cells were filtered and resuspended in fresh trypticase soy broth where they grew and multiplied. This indicated that the physical stability of *C. echinulata* was in excess of 21 days compared to 7 days for free cells.

Two types of fluidized bed tower reactors were used in these studies. The fluidized bed reactor with an external loop for aeration is shown in Fig. 7 . The reactor was comprised of a Pharmacia C26/100 glass column with a JC

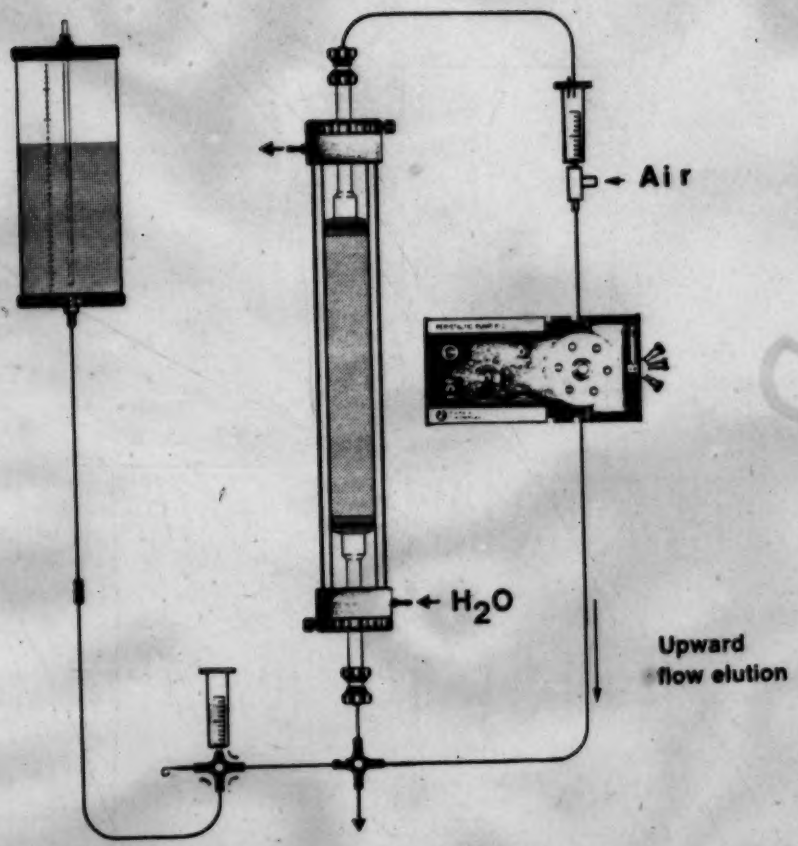


Figure 7 . Fluidized bed tower reactor with upward elution using an external circulation loop for aeration. The air lift reactor does not utilize the external loop.

26/100 water jacket and two AC 26 adaptors. Aeration of the medium was accomplished in a aeration chamber (plastic syringe without plunger) attached to a Pharmacia LV4 four way valve. The design allowed a narrow gauge tube to pass through a rubber septum and extend fully into the syringe. Back pressure was prevented by venting the gas through a 21 gauge needle attached to the rubber membrane at the top of the syringe. Elution was upward and aeration was with either pure oxygen (Linde) or compressed air (Linde). Gas flow was regulated by a fine metering needle valve. Media flow through the system was controlled by a Pharmacia P3 peristalic pump set at 140 mL/h.

A second fluidized bed tower reactor was designed to incorporate air lift into the system to recirculate the medium in a closed column (Fig. 7). The gas flow was regulated by a fine metering needle valve and kept at a pressure sufficient to recirculate the immobilized cells and the medium. Gas pressure was vented through the upper AC 26 adaptor which was set so as not to become plugged by mycelium and foaming medium.

Immobilized or free cells were added to the reactors using standard aseptic technique.

The contents of the air lift reactor containing *T. sporulosum* (approximately 175 mL) were extracted successively at pH 5.5, pH 1 (HCl) and pH 9.5 (potassium carbonate) with two 50 mL volumes of ethyl acetate. The three extracts were separately reduced in volume to about 2

mL and analyzed by TLC.

3.5.3 Microbiological Assay

The bacteria used for this assay, *Klebsiella morganii* B23, *Salmonella typhimurium* ATCC 6539 and *M. smegmatis* ATCC 14468 were transferred from antibiotic medium A (BBL) slants to antibiotic medium D broth (BBL) and incubated overnight at 37°C with shaking at 250 rpm. A 1 mL aliquot of this suspension was added to 200 mL of antibiotic seed agar A and dispensed into standard petri plates (15 mL/plate). The fungi, *Neurospora sitophilla* UAMH 3501 and *Rhodotorula glutinis* UAMH 2660 were started on slants and spores were then transferred to Sabouraud dextrose medium and incubated overnight at 27°C on a gyratory shaker, 250 rpm. This is the optimal growth temperature for these organisms. A 1 mL aliquot of these cultures was dispersed in 100 mL of Sabouraud dextrose agar and plated as described above. All plates were stored at 4°C until used.

Schleichen and Schuell 12.7 mm absorbent paper discs were impregnated with 100 uL of test solution. The damp discs were applied directly to the agar plates. *K. morganii*, *S. typhimurium* and *M. smegmatis* were incubated at 37°C and *N. sitophilla* and *R. glutinis* were incubated at 27°C. Penicylinders were arranged on plates equidistant from each other to minimize zone overlap. A 0.1 mL aliquot of test solution was added to each cylinder. Zones of inhibition were measured as the diameter in mm units.

3.6 1,2-Diketone Analysis

3.6.1 Diacetyl Assay Calibration Graph

A series of dilutions was prepared from a 10 mg/L stock solution of diacetyl. Samples, 1 mL, were added to tubes containing 0.1 mL of 0.1% *o*-phenylenediamine (OPA) (R.T. Coutts, personal communication) and 0.1 mL of 0.1% anthracene (internal standard) in methylene chloride. The samples were acidified with 0.1 mL of 13% HCl and allowed to react for 30 min with occasional shaking. The samples were extracted twice with two 5 mL volumes of ether-methylene chloride (55:45 v/v). The extracts were combined and reduced in volume to about 50 uL at 55°C prior to GLC analysis. The standard curve comparing the ratio of diacetyl and internal standard to the concentration of diacetyl in the sample was linear between the tested range, 1.0 and 10.0 ug.

3.6.2 Acetoin Assay Calibration Graph

Acetoin does not form a single product with OPA unless it has been oxidized to the diacetyl prior to OPA treatment.

A 1 mL aliquot of dilutions prepared from a 10 mg/L stock solution of acetoin was added to 2.5 mL of a 12% solution of ferric chloride in a sealed tube, boiled for 15 min, cooled at 4°C, basified and extracted twice with two volumes of ether-methylene chloride. The extracts were combined with OPA and internal standard and acidified as detailed in the diacetyl assay. The solution was vortexed

for 1 min and centrifuged. The solvent layer was removed and the sample extracted a second time with 2 mL of fresh solvent. The solvent layers were combined and reduced in volume to 50 uL at 55°C. The standard curve comparing the ratio of acetoin (present as diacetyl) and internal standard with the known concentration of acetoin was linear within the range of 1.0-10.0 ug.

3.6.3 Ketone Analysis

Analysis of microbial incubation broths or malt products for the presence of acetoin and diacetyl was performed in the manner prescribed in the above sections. The assay was performed in 12 mL conical test tubes with teflon stoppers containing sodium chloride (200 mg) to reduce emulsion formation.

3.7 Substrates and Reference Materials

Many of the amphetamine, phenylethylamine and phentermine substrates studied had been prepared by, or with the cooperation of Dr. R.T. Coutts prior to the initiation of this study (Beckett *et al.* 1973a,b; Coutts, Dawson and Beckett 1976; Coutts, Dawson *et al.* 1976). Chemical synthesis procedures of the metabolites and substrates prepared during this study is included for reference purposes.

3.7.1 N-Acetylated Derivatives

N-acetylamphetamine was formed by the addition of excess acetic anhydride to amphetamine base in ether at 4°C. Filtration gave the N-acetylated product which was recrystallized from ether-n-pentane, mp 93-95°C.

The N-acetyl derivatives of *p*-chlorophenylethylamine, *p*-chloroamphetamine, phenylethylamine, phenelzine, pheniprazine, 2-phenylcyclopropanamine and other substrates were prepared by aqueous acetylation of the parent molecule under alkaline conditions (Method C, section 3.4.1). Each product of this reaction was of sufficient purity for GLC analysis. In addition, acetylated derivatives were routinely prepared by one of the procedures outlined in section 3.5.1.

3.7.2 Acetoin Conjugates

The PCA and AMPH conjugates (4-chloro-*alpha*-methyl-N-(1-methylacetonylidene)benzeneethanamine, 4-chloro-*alpha*-methyl-N-(1-methylacetonyl)benzeneethanamine, *alpha*-methyl-N-(1-methylacetonyl)benzeneethanamine, and *alpha*-methyl-N-(1-methylacetonyl)benzeneethanamine, respectively) were synthesized by reacting PCA or AMPH free base (100 mg) with acetoin (100 mg) in 1 mL of benzene containing 1 g of 4A molecular sieves (4-8 mesh). The mixture was heated under reflux for 5 min and then allowed to stand at room temperature. The mixture of products thus obtained was analyzed by GLC/MS after alkaline extraction (Method A,

section 3.4.1).

A 0.1 mL portion of the benzene solution containing these products was reduced to dryness and the products were dissolved in 0.2 mL absolute ethanol. The ethanolic solution was treated with 1 mg of SBH and monitored by GLC/MS. The aliquot (10 uL) removed for analysis was treated with 1% HCl to stop the reaction and then extracted by Method A (section 3.4.1).

3.7.3 Diacetyl Conjugates

The diacetyl conjugates of amphetamine, *alpha*-methyl-N-(1-methylacetonylidene)benzeneethanamine and *p*-chloroamphetamine, 4-chloro-*alpha*-methyl-N-(1-methylacetonylidene)benzeneethanamine were synthesized by reacting the respective free base (100 mg) with 100 mg of diacetyl in 5 mL of benzene containing 1 g of 4A molecular sieves (4-8 mesh). The solution was heated under reflux for 5 min and allowed to equilibrate to room temperature. The reaction was monitored by GLC/MS analysis (Method A, section 3.4.1).

3.7.4 4-Hydroxy-2-butanone Conjugate

The 4-hydroxy-2-butanone conjugate of *p*-chloroamphetamine was prepared by a two step process. The first step involved formation of the imine by reacting 100 mg of the free base of *p*-chloroamphetamine with 100 mg of 4-hydroxy-2-butanone in 5 mL of benzene containing 1 g of 4A molecular sieves (4-8 mesh) at room temperature. The

reaction was monitored by GLC/MS and was stopped when *p*-chloroamphetamine was no longer detectable in the reaction mixture. The molecular sieves were filtered off and washed with two 5 mL portions of benzene. The combined benzene fraction was reduced to dryness and the residue was dissolved in 5 mL of absolute ethanol. The ethanolic solution was treated with 20 mg of SBH for 15 min. The reaction was stopped and analyzed as outlined in section 3.4.4.

3.7.5 p-Hydroxyphentermine

1-*p*-Methoxyphenyl-2-nitro-1-propene was obtained by the reaction of *p*-methoxybenzaldehyde and nitroethane (Pearl and Beyer 1951). Reduction of the resulting nitroolefin with zinc-acetic acid (50 g zinc powder in 40 mL water and 400 mL glacial acetic acid) gave *p*-methoxyphenylacetone (Dodson and Riegel 1948). Methylation of 9 g of the above ketone in diethyl ether with Grignard reagent (19.5 g iodomethane and 10 g magnesium in 150 mL ether) gave *p*-methoxyphenyl-2-methyl 2-propanol (Data *et al.* 1966). Reaction of this alcohol with potassium cyanide in a mixture of sulfuric and acetic acid gave the corresponding acetamide which was then hydrolysed by heating under reflux with 50 mL 19% HCl to give *p*-methoxyphentermine. The free base was generated from the HCl salt and then treated with 48% HBr to cleave the methyl ether. *p*-Hydroxyphentermine was isolated as the HBr salt.

3.7.6 N-(Isopropyl)phentermine

Acetone (2.4 mL) and phentermine (1 g) were reacted in 10 mL of diethyl ether containing 10 g of 4A molecular sieves 4-8 mesh) at room temperature. The reaction was monitored by GLC and was stopped when the formation of the resulting imine was complete. The ether was removed by filtration and the molecular sieves were washed once with a 5 mL aliquot of ether. The combined ether extract was reduced to dryness and dissolved in 5 mL of absolute ethanol and reacted with 50 mg of SBH. The reaction was monitored and worked up as described in section 3.4.4. The HCl salt was recrystallized from acetonitrile, mp 178.5-179°C. The reported value is 176-178°C (Schulz 1967).

3.7.7 N-(n-Propyl)phentermine

This compound was prepared by the LAH reduction of N-propionylphentermine (prepared by Dr. G. Jones). To a 10 mL diethyl ether suspension of 0.30 g of LAH, the imine (0.8 g) in 20 mL of ether was added dropwise. The HCl salt of the resulting base was recrystallized from acetonitrile, mp 200°C, identical to the reported value (Data *et al.* 1966).

3.7.8 N-Propargylbenzylamine

N-Propargylamine was synthesized by the reaction of 2.12 g of benzaldehyde with 1.65 g of propargylamine in 50 mL of diethyl ether containing 20 g of 4A molecular sieves (4-8 mesh). The molecular sieves were filtered off and

washed with two 50 mL volumes of ether. The combined ether fractions were reduced in volume to approximately 50 mL and treated with LAH (0.8 g) (portion wise). The reaction was stopped after 1 h by the addition of 100 mL of 20% aqueous sodium hydroxide. N-Propargylbenzylamine was extracted (Method A, section 3.4.1) and the solvent reduced to dryness.

3.7.9 N-Hydroxy-N-propargylbenzylamine

N-Propargylbenzylamine (200 mg of the free base) was treated with 30% hydrogen peroxide (2 mL) in acetone (2 mL) for 24 h at room temperature. The reaction was monitored by GLC/MS and the yield of product was 48%, together with 40% of starting material. The title compound could be separated from starting material by TLC using silica gel G developed in benzene-diethyl ether (1:1, v/v). The elution time (Rf) of N-hydroxy N-propargylbenzylamine was 0.65. The product was labile and not purified.

3.7.10 N-(Isopropyl)phenylethylamine

Acetone (12.4 mL) and phenylethylamine (4.13 g) were reacted in diethyl ether (50 mL) with 3A molecular sieves (4-8 mesh) (40 g) as a dessicant. The ether was evaporated *in vacuo* and the imine was redissolved in 10 mL of absolute ethanol and reacted with 1.0 g of SBH (section 3.4.4). The compound was recrystallized from acetonitrile as the HCl salt, mp 166-166.5°C. The reported literature value is

168-169°C, from acetonitrile (Shapiro *et al*. 1959).

3.7.11 N,N-(Di-n-propyl)phenylethylamine and N-(n-Propyl)phenylethylamine

The free base of phenylethylamine (0.49 g) was reacted with N-propyl bromide (0.492 g) in a 5 mL saturated solution of potassium carbonate containing 5 mL of tetrahydrofuran. The reaction mixture was heated under reflux for 39 h and purified using silica gel column chromatography developed with diethyl ether. The first 50 mL were discarded, the second 50 mL contained N,N-(di-*n*-propyl)phenylethylamine. An additional 175 mL of ether was required to recover N-(*n*-propyl)phenylethylamine. The HCl salts were prepared and recrystallized from acetonitrile: (NPE) mp 217-217.5°C, reported 215-216°C (Shapiro *et al*. 1959) and acetonitrile-ether (DPE), mp 103-103.5°C, no reported value for the HCl salt.

4. Results and Discussion

4.1 Introduction

Extracts of incubation broths containing micro-organisms, drug substrates and their metabolites, and control broths to which substrates but no micro-organisms had been added were examined by GLC. Identification of metabolites was generally based on comparison of their GLC retention times and mass spectra with those of authentic reference compounds. In some instances, identification of some metabolites or their chemical derivatives was based on interpretation of MS fragmentation patterns.

Different extraction procedures were required because of the dissimilar solubility characteristics of the metabolites. All incubation broths in this study were assayed by extraction from alkaline solution (Method A, section 3.4.1) in order to recover neutral and basic metabolites, including unmetabolized substrate. Carboxylic acid metabolites were extracted from acidic solution (Method B) and esterified with chloroethanol/HCl to facilitate GLC-MS analysis. Chlorine containing fragments in the mass spectra were easily identified because of the natural isotopic abundances of ^{35}Cl and ^{37}Cl. This extraction method was necessary only in those studies using phenylethylamine compounds or *p*-chloroamphetamine as substrates. Since direct extraction of phenolic metabolites of phenylethylamine and

amphetamine from acidic or alkaline solution was not efficient because of their amphoteric nature, these metabolites were acetylated in aqueous solution (Method C) prior to extraction (Coutts *et al*. 1979, 1980). Acetic anhydride reacts readily with phenols, primary and secondary amines and substituted hydrazines, but not with alcohols, in aqueous media to form the mono- or di-acyl derivatives. The resulting neutral acetates had a reduced water solubility and their extraction efficiency was greatly improved.

Analysis of drugs in biological fluids requires confirmation that no other substances interfere with the analysis or react with the substrate or its metabolites. This necessitated that the solvents were distilled prior to use and that the solvent controls were tested to ensure that impurities which might react with the substrate were absent. The mixed solvent used in this study, diethyl ether-methylene chloride was formulated to obtain optimal partitioning of substrates and metabolites into the organic solvent (Siek 1978). The partitioning of drugs and their metabolites was influenced by the ionic strength and pH of the aqueous phase. To promote recovery of these compounds, excess sodium chloride was added to the aqueous samples and the pH was suitably modified prior to extraction.

It was evident that with few exceptions, all possible metabolites could not be detected on any one GLC system without interference from endogenous media and microbial compounds. The interference from these compounds was

particularly noticeable when the solvent extracts were derivatized with chloroethanol or TMS reagents.

It was possible, in principle, to quantitate the absolute amounts of all metabolites detected in this study. Several metabolites, however, were labile and authentic standards for some N-oxidation and ring hydroxylation products were not available. An additional obstacle to absolute quantitation was the variety of media used, which influenced extraction efficiency. Therefore, quantitation of metabolites using calibration curves was not attempted. Quantitation of metabolites in relative amounts was possible, however, by comparison of relative peak areas of the GLC traces of recoverable substrate and metabolites. Various GLC columns were investigated to ensure that the peaks recorded by this method could be detected on one column and were free from interfering contaminants.

4.2 Screening Experiments

The initial screening experiments were conducted with *Aspergillus niger* ATCC 9142, *A. ochraceus* ATCC 1008, *Cunninghamella echinulata* ATCC 3655 and 9244, and *Streptomyces platensis* ATCC 13865 using submerged cells in a modified trypticase soy broth. Metabolism of (±)-N-(*n*-propyl)amphetamine (NPA) by *C. echinulata* ATCC 9244 and ATCC 3655 produced several metabolites (Table 6). With one exception, all were known mammalian metabolites of NPA

Table 6 . Relative recoveries of substrate and metabolites of NPA in the initial fungal screen.

Organism	% Yield[1]				
	AMPH	NPA	AcAMPH	NOX	pOH-NPA
ATCC 1008	tr[2]	100	-	-	-
ATCC 3655	1.3	78.4	4.1	8.1	8.1
ATCC 9142	-	100	-	-	-
ATCC 9244	58.7	25.7	5.8	3.2	6.6
ATCC 13865	1.2	78.1	4.4	5.5	10.8
Control	-	100	-	-	-

[1] Based on relative peak areas.

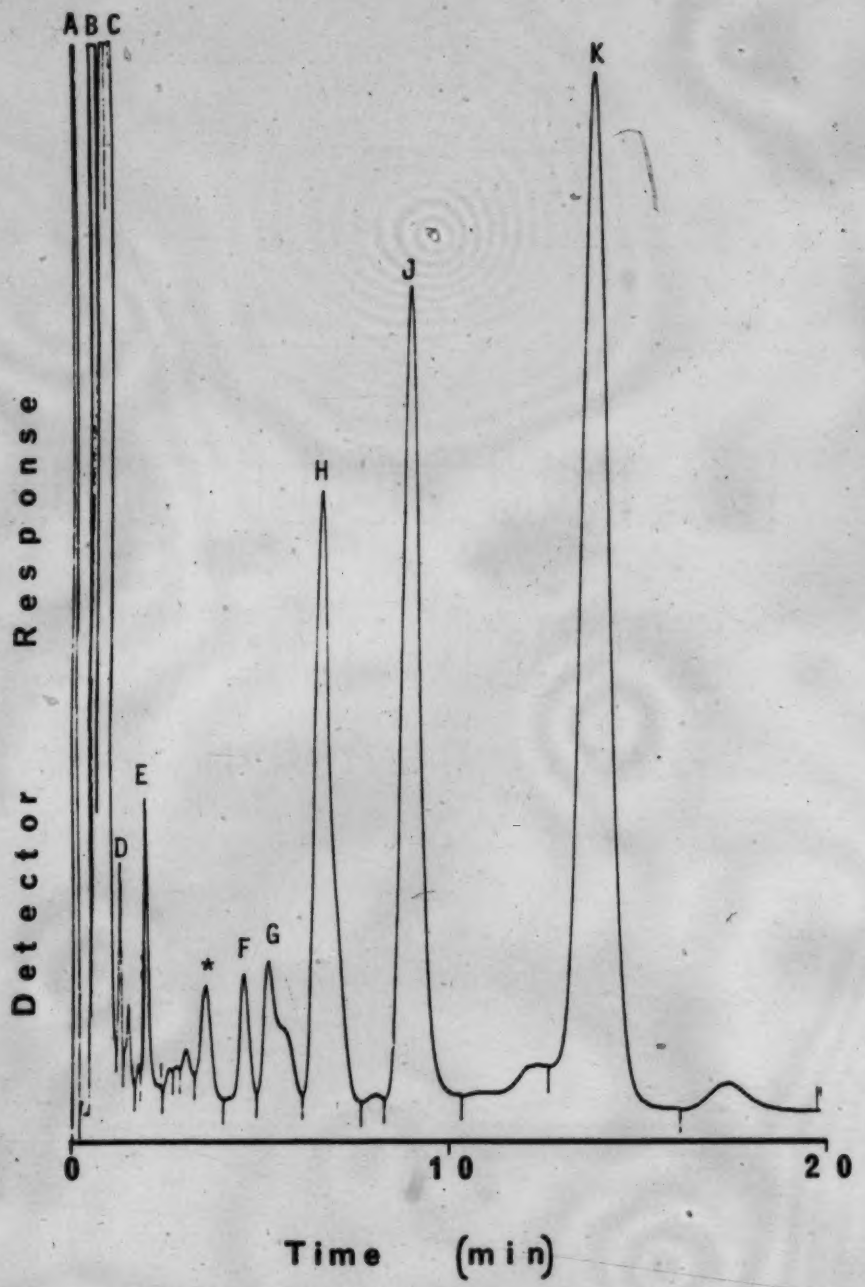
[2] tr, trace.

(Coutts and Dawson 1977; Coutts, Dawson *et al*. 1976). Compounds F, G, H, and J (Fig. 8) were the N-oxygenated products N-hydroxyamphetamine (NOH-AMPH), N-hydroxy-N-(-*n*-propyl)amphetamine (NOH-NPA), 1-phenyl-2-propanone oxime (OXM) and *alpha*-methyl-N-(*n*-propylidene)benzeneethanamine N-oxide (NOX), respectively. Compound C is NPA.

To minimize decomposition of metabolically produced hydroxylamines, the incubation medium was buffered to pH 7.4 and metabolites were extracted at this pH value and from samples basified immediately prior to extraction. Glass GLC columns were used throughout to minimize "on-column" degradation of N-oxidation products. In control experiments, NOH-NPA decomposed to NOX to a minor extent (5%) but NOH-AMPH decomposition resulted in a 35% degradation to OXM. Compared to the quantities of N-oxidized products isolated from the control experiments, the relative amounts of N-oxidation products detected in the metabolic extracts suggest that both chemical and biological processes are involved in the formation of OXM and NOX.

Compound B was the major metabolite, based on its GLC peak area, and was identified as amphetamine (AMPH)(Coutts and Beckett 1977). Comparison of the mass spectrum of metabolite L with the spectrum of an authentic sample indicated that it was the mono-oxygenated derivative of the NPA substrate, *p*-hydroxy-N-(*n*-propyl)amphetamine (*p*OH-NPA), which is also a major *in vivo* metabolite in rat (Coutts, Dawson *et al*. 1976). This metabolite was not detected by GLC

Figure 8 . GLC trace of metabolites formed from *C. echinulata* ATCC 9244 biotransformation of NPA. System B, 145°C. * unidentified metabolite.

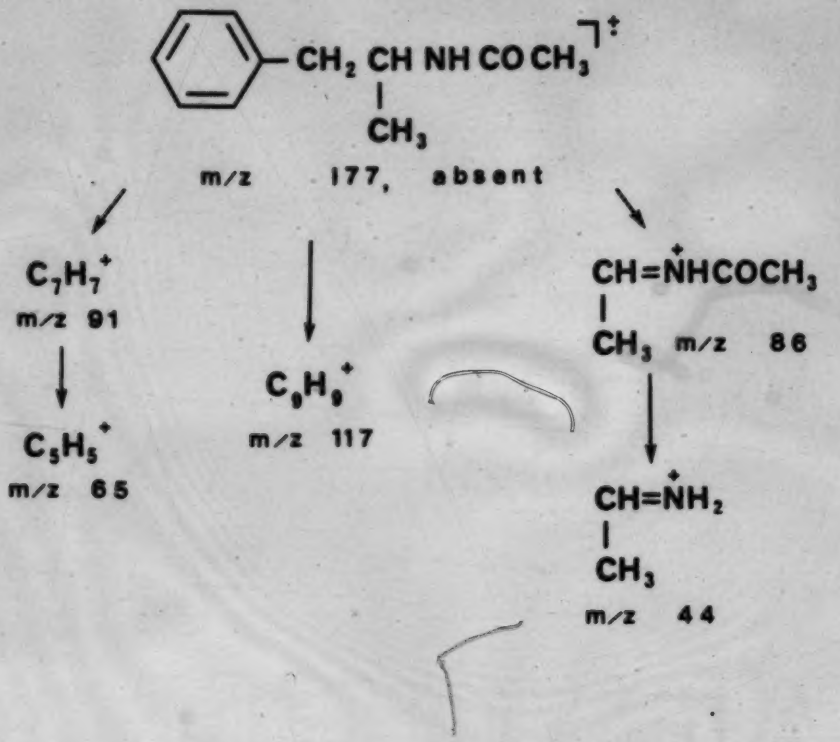


analysis on system B (Fig. 8) but is readily observed on system A.

Three minor metabolites of NPA (A, D and E) were identified on the basis of GLC/MS analysis as benzyl alcohol, 1-phenyl-2-propanone and the corresponding alcohol, 1-phenyl-2-propanol. These compounds are all known mammalian products of NPA biotransformation, but metabolite K is not. Diagnostic ions in the spectrum of K are consistent with those expected for N-acetylamphetamine (AcAMPH) (Fig. 9). Authentic AcAMPH was synthesized and found to have GLC/MS properties identical to K.

4.3 Incubation Conditions and Media Selection

Smith and Rosazza (1975) described several media suitable for fungal biotransformation studies but because of the complex and generally irreproducible nature of these media, several commercial media were tested in an attempt to find a more reproducible medium capable of supporting the growth of a variety of micro-organisms. The soya bean meal and corn steep liquor recommended by these authors were formulated and tested against Mueller Hinton, trypticase soy broth, brain heart infusion, nutrient broth, Sabouraud dextrose and modified trypticase soy broth for their ability to support *C. echinulata* biotransformation of NPA. The modified trypticase soy medium permitted the highest transformation of NPA and recovery of identifiable metabolites. The soy



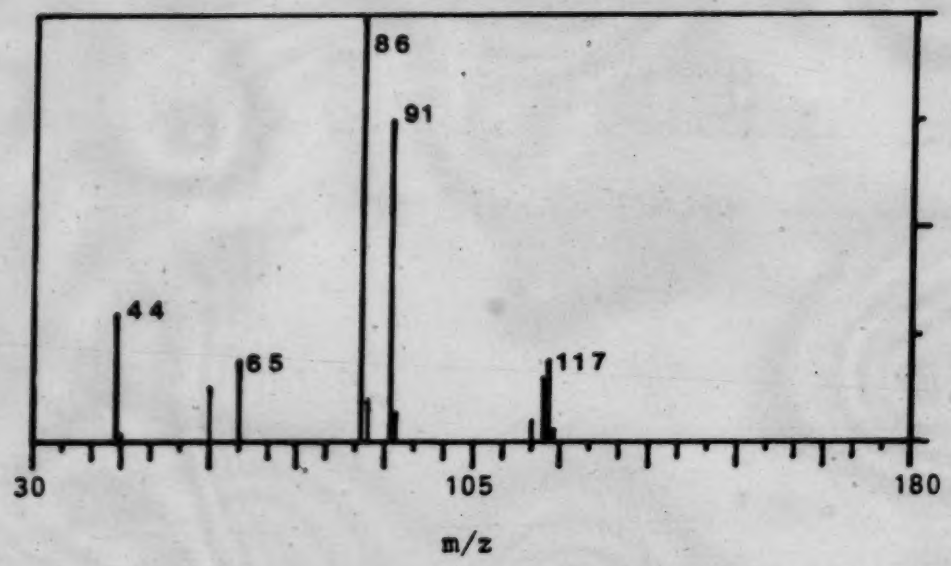


Figure 9 . Mass spectral fragmentation pattern of N-acetyl-amphetamine detected in *C. echinulata* metabolic extracts.

broth and corn steep liquor media reported by Smith and Rosazza (1975) supported a high rate of N-dealkylation but had noticeably reduced amounts of the N-oxidation products.

Stationary cultures of these organisms were analyzed after 14 days in stage II to determine the effect of reduced aeration on biotransformation. GLC analysis revealed that no transformation of NPA to detectable (or identifiable) products occurred with ATCC 1008, 3655 and 9142. *C. echinulata* ATCC 9244 and *Str. platensis* ATCC 13865 were capable of N-dealkylation, N-acetylation and ring hydroxylation; the former organism was the more efficient. No N-oxidation products were detected in this study.

In submerged cell culture, the time of appearance of each metabolite varied, AMPH was detected within 24 hour of substrate addition, followed by formation of NOH-NPA, NOX, AcAMPH and OXM. The recovery of these products was low until day 5 when synthesis increased up to day 7. In *C. echinulata* cultures grown using a modified trypticase soy broth, the pH dropped to 6.1 after 48 hours and then went to pH 8.2 after 96 hours. The biomass and growth characteristics of *C. echinulata* were dependant on the quality and quantity of inoculum. A 5-10 % inoculum produced a thick heavy growth of long filamentous mycelia which coalesced to form a single macro pellet when flasks were incubated in the flat mode. Such a macro pellet would severely restrict nutrient diffusion and oxygen availability (Righelato 1975). To enhance oxygen transfer, cultures were incubated on a shaker

equipped with 45° angle brackets. High amounts of inoculum still resulted in formation of a macro pellet, but a range of 3-5% enabled the cells to grow as discrete filaments. The apparent viscosity within the flask was high but the cells were able to circulate freely. The results of incubating *C. echinulata* with varying amounts of dextrose in either the flat (180°) or raised (45°) mode are given in Table 7 . There was little effect on N-dealkylation, but N-acetylation decreased with increasing dextrose levels. Formation of *p*OH-NPA was influenced by the amount of dextrose added and the method of shaking the incubation flasks. The use of the angle brackets was found to enhance the formation of metabolites and was accordingly used for all subsequent studies.

Colony morphology in the submerged system was also influenced by the incubation temperature as cells grown at 27° in trypticase media were filamentous whereas the corresponding cultures at 37°C were observed to be micro pellets.

The time of substrate addition to stage II cultures was significant (Table 8). The most appropriate time to add substrate was dependant on the amount of dextrose added at the start of stage II growth. Cellular mass increased rapidly in stage II and being a biological process, caused permanent changes to the medium. The colour of the medium and cells went from light yellow initially to a medium brown after 6 days. During this transition the apparent viscosity increased to a highly viscous fluid and then decreased with

Table 7 . The effect of dextrose concentration and shaking parameters on the biotransformation of NPA by *C. echinulata*.

		% Yield[1]					
Mode	Dextrose[2]	AMPH	NPA	NOH	OXM	NOX	AcAMPH
45°	-	15.8	70.7	1.7	1.7	6.3	3.8
45°	1 mL	28.0	65.9	-	1.3	1.3	3.5
flat	-	25.9	67.2	1.1	2.2	1.1	2.5
flat	1 mL	35.7	57.1	1.1	1.4	1.1	3.6
flat	3 mL	27.7	70.9	1.4	-	-	-
flat	5 mL	29.1	70.9	-	-	-	-

[1] Based on relative peak areas. [2] Dextrose was prepared as a 40g/L solution.

Table 8 . The effect of dextrose concentration and time of substrate addition on the biotransformation of NPA by *C. echinulata*.

		% Yield[1]					
Time[2]	Dextrose[3]	AMPH	NPA	NOH	OXM	NOX	AcAMPH
48 h	1 mL	25.6	66.9	0.8	1.7	0.8	4.2
48 h	3 mL	94.6	-	1.1	1.1	1.1	2.1
48 h	5 mL	54.6	42.3	1.4	-	1.7	-
72 h	1 mL	-	96.1	1.3	1.3	0.8	0.5
72 h	3 mL	43.3	49.5	0.5	3.5	1.5	1.7

[1] Based on relative peak areas. [2] Time of substrate addition to stage II growth. [3] Dextrose prepared as 4% w/v solution.

colour formation to a watery mixture of intact and lysed cells.

The metabolic profile could be altered by the choice of carbon source and the nature of the medium used. Stage I *C. echinulata* was grown and homogenized in the trypticase soy medium and added to flasks containing one of three carbon sources in either 5 % (minimal) or full strength media. Growth was poor when *C. echinulata* was incubated in the minimal medium containing citrate but a heavy growth of micro pellets was observed in the regular strength medium. Saccharose and dextrose elicited the same responses with pellet formation in the minimal media and heavy filamentous growth in the full strength media. Under these conditions, saccharose was the favoured carbon source, as observed by the level of NPA transformation and only minor quantities of metabolites were detected in either medium containing citrate.

4.3.1 Strain Degeneration

The phenotypic expression of N-oxidation was readily lost by this organism. The culture obtained from ATCC at the start of this project was able to effect high rates of N-oxidation and ring hydroxylation but after two months the expression was lost in the running culture of *C. echinulata*. Continued re-culturing of this organism resulted in re-expression of this characteristic but at a greatly reduced level. A second culture was obtained from ATCC. This

culture was contaminated with an unknown bacterium and did not synthesize significant amounts of the N-oxidation products when it was freed of this contaminant. Subsequently a third culture was obtained which did mediate formation of the profile observed with the first strain. Unfortunately, the level of N-oxidation was considerably less than that observed with the initial culture. Repeated culturing did, however, increase the expression of this phenotype to the point where it was acceptable for further studies.

During the period of time required to re-establish the ATCC culture as the working organism, strains of *C. echinulata* were obtained from Dr. R. Haskins, Prairie Regional Laboratory, Saskatchewan, (PRL 28 and 32) and Dr. J. P. Ferris, Rensselaer Polytechnic Institute, New York, USA, (Ferris A and B).[1] The comparison of these strains with those from ATCC are presented below:

1. ATCC 9244 (original culture). This culture was observed as a mixture of 2-6 mm pellets and the final pH was 8.5. AMPH and *p*OH-NPA were the major products with only trace amounts of NOX and AcAMPH detected.
2. ATCC 9244 (second culture). This culture grew as a single mat of thick mycelia, the final pH was 8.3. NPA was recovered in near quantitative amounts with only traces of AMPH, AcAMPH, NOX and *p*OH-NPA detected.
3. ATCC 9244 (third culture). The findings for this culture

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[1]Dr. Ferris mentioned that his cultures were subject to loss of transformation phenotypes.

were identical to those of the second culture.

4. Ferris A. Pellets of 2-6 mm were present in the submerged culture; the final pH was 8.3. The biotransformation profile was identical to that of the original ATCC culture.
5. Ferris B. This culture grew as a thick mycelial mass having a final pH of 8.4. The metabolic pattern was similar to that of the second and third ATCC cultures but with a higher proportion of N-oxidation products.
6. PRL 28. This culture grew as micro pellets, the solution having a final pH of 8.6. The spectrum of products detected in the extract was similar to that of the original ATCC culture.
7. PRL 32. The physical and metabolic pattern was identical to that of PRL 28.

The variation in the growth pattern was interesting as all growth factors were standardized to ensure that the size and age of inoculum was consistent in all strains. This would indicate that the differences were due to genetic and not environmental factors.

4.3.2 UAMH Fungal Screen

Several of the cultures used in this study (Table 2) grew poorly in modified trypticase soy medium and could not be transferred to stage II conditions on day 3 but were transferable by day 5. Cultures that did mediate biotransformation were found to effect only one or two

transformations in low yield (Table 9). UAMH 3015 was the exception in that N-dealkylation accounted for over 75% of the recoverable products.

4.3.3 Bacterial and Actinomycete Screen

The microbial metabolism screen of NPA biotransformation was extended to bacterial and actinomycete cultures. These organisms were incubated in nutrient broth supplemented with dextrose overnight, filtered, resuspended in minimal medium and incubated at 37°C. Samples were removed at days 1, 3 and 5 of stage II and extracts were analyzed by TLC on silica gel G, solvent system A (section 3.4.8). The plates were visualized by long and short wave UV, iodine vapour and spraying a duplicate with Dragendorff reagent. *Mycobacterium smegmatis* was the only organism tested that produced detectable quantities of N-oxidation products and was chosen for future studies on this basis.

GLC examination of the extract from the *M. smegmatis* incubation broth containing NPA revealed the presence of three peaks, designated as A-C (Fig. 10). Compound A was readily identified as the N-dealkylation product AMPH and C was determined to be the corresponding N-acetyl derivative AcAMPH. Metabolite B was identified as the nitrone, NOX.

Table 9 . Relative recovery of NPA and NPA metabolites in the major fungal screen.

Organism	% Yield[1]					
	AMPH	NPA	NOH	OXIME	NOX	AcAMPH
UAMH 2937	tr[2]	100	tr	-	tr	-
UAMH 202	tr	100	tr	-	tr	-
UAMH 1848	2.7	97.3	tr	-	tr	tr
UAMH 2170	5.1	92.0	tr	2.9	tr	-
UAMH 485	2.4	97.6	tr	-	-	-
UAMH 1365	5.2	93.0	tr	1.2	-	tr
UAMH 1947	-	100	tr	tr	tr	-
UAMH 199	-	100	tr	-	tr	-
UAMH 3585	tr	96.7	3.3	-	tr	tr
UAMH 1406	4.8	83.7	tr	4.9	2.4	4.2
UAMH 3477	2.6	89.5	tr	tr	7.9	-
UAMH 3015	69.6	22.9	tr	tr	tr	7.5
UAMH 1615	1.8	95.8	2.4	tr	tr	tr
UAMH 1952	2.3	94.9	tr	-	tr	2.8
UAMH 1793	1.5	98.5	tr	-	tr	tr

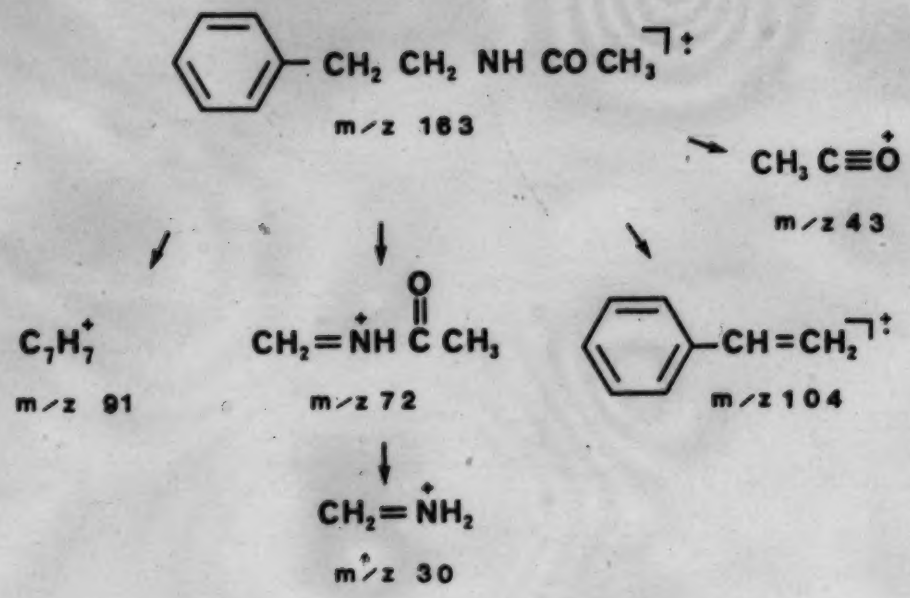
[1] Based on relative peak area. [2] trace.

Figure 10 . GLC trace of the metabolic profile obtained from *M. smegmatis* biotransformation of NPA. System B, 145°C.

4.3.4 Clinical Isolates

There are only two reports dealing with the anaerobic biotransformation of amphetamine-like compounds (Caldwell and Hawksworth 1973; Caldwell *et al.*, 1972). In general, the types of organisms found in the gastro-intestinal tract of laboratory animals are similar to those observed in man (Savage 1977; Scheline 1973). However, distribution throughout the tract is different. The major reaction observed was the N-dealkylation of NMA by various enterococci, lactobacilli and clostridia with little or no N-demethylation in the enterobacteria, bacteroides or bifidobacteria. The present study was undertaken to determine the factors necessary for further biotransformation of amphetamine homologues in representative cultures of clinical isolates.

Cultures provided by the University of Alberta Hospital (UAH) were first cultures after clinical isolation (Table 4). Immediately upon receipt a 1 mL aliquot of each culture was added aseptically to sterile, freshly degassed medium containing methylene blue as oxygen indicator. Cultures were incubated and analyzed at days 7 and 14. Only cultures which did not oxidize methylene blue to its green oxidation product were tested further. Bacteroides A17069 was the only culture with a detectable product which was not also in the medium controls. The metabolite was readily identified as the N-acetylation product of phenylethylamine, N-acetylphenylethylamine (AcPHE, Fig. 11).



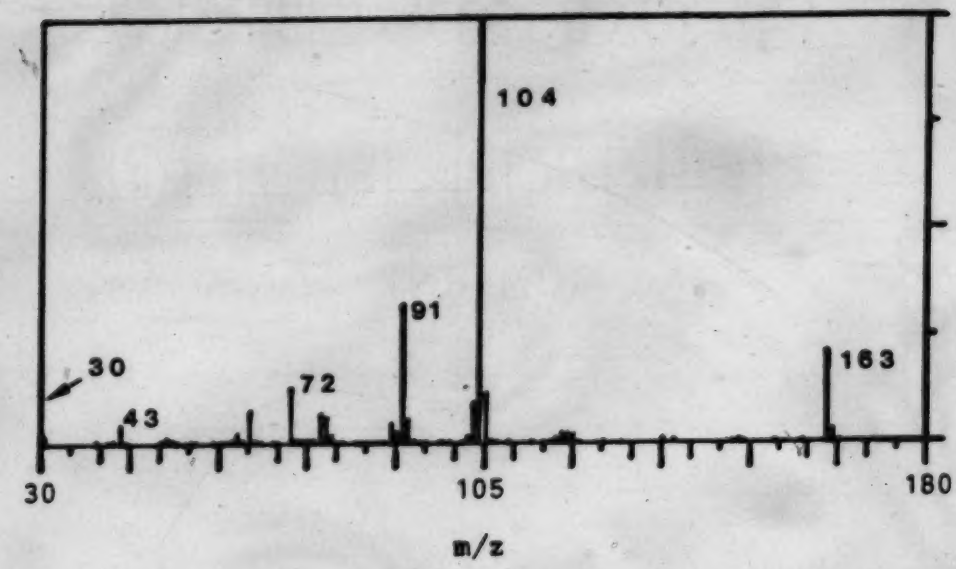


Figure 11 . Mass spectral pattern of N-acetylphenylethylamine.

This compound would not be formed by the C and N demethylation of NMA and the study was repeated with additional controls. A17069 was then found to be able to mediate the formation of this product from endogenous medium constituents. It was confirmed that the medium did not contain AcPHE; however PHE was present in detectable amounts. This suggested that A17069 could effect N-acetylation of a primary amine but as this was not the focus of the study further experiments with clinical isolates were not performed.

4.4 Phenylethylamine series

GLC analysis of the alkaline extracts (Method A, section 3.4.1) of *C. echinulata* incubations with β-phenylethylamine (PHE) revealed a single metabolite identified as N-acetyl-β-phenylethylamine (AcPHE) (Table 10). Extraction methods B and C did not reveal additional metabolites even though AcPHE accounted for less than 30% of the added substrate. The expected product of oxidative deamination, phenylacetic acid, was not observed presumably as a result of further oxidation to undetectable metabolites (Hoffman and Rehm 1978).

A recent report (Haller and Finn 1979) describing the resistance of *p*-chlorophenylacetic acid to oxidative metabolism prompted a study of *p*-chlorophenylethylamine (CLE). Incubation broths containing *C. echinulata* and CLE

Table 10 . Metabolic patterns observed in the biotransformation of phenylethylamine and its homologues by *C. echinulata*.

Substrate	Metabolic patterns[1]				
	OD	ND	NO	NAc	pOH
PHE	-	NA[2]	-	+++	-
pCl-PHE	++	NA	-	++	-
NPE	-	+	-	+++	-
DPE	-	+	-	+++	-
IPE	-	+	-	+++	-
AMPH	-	NA	+	+++	+
CLA	-	NA	-	+++	-
NPA	-	++	+++	+++	+++
IPA	-	++	++	+++	+++
PHT	-	NA	-	-	+++
NPT	-	+	-	-	+++
IPT	-	+	-	-	+++

[1] (-) no metabolite observed, (+) trace metabolite, (++) minor metabolite, (+++) major metabolite. (OD) oxidative deamination, (ND) N-dealkylation, (NO) N-oxidation, (NAc) N-acetylation, (pOH) ring hydroxylation, [2] (NA) not applicable.

did indeed contain *p*-chlorophenylacetic acid. This metabolite was determined as the chloroethyl ester (Method B) and characterized on the basis of its mass spectrum (Fig. 12). N-Acetyl-*p*-chlorophenylethylamine (AcCLE) was also detected as a minor metabolite (Fig. 13). On the basis of this evidence it is conceivable that phenylacetic acid is a metabolite of PHE but its detection is difficult due to rapid incorporation into the intermediary metabolic pathways of these organisms.

M. smegmatis incubations broths containing PHE were totally devoid of detectable substrate and metabolites (Table 11). *M. phlei* is known to incorporate both benzoic and phenylacetic acids into its metabolic pathways (Guerin *et al*. 1967). Catabolism of substituted benzoic acid has also been reported in species of Nocardia (Klages and Lindens 1979) and Streptomycetes (Sutherland *et al*. 1981). Analysis of *M. smegmatis* incubation broths (Method B) containing CLE revealed the presence of the chloroethanol ester of *p*-chlorophenylacetic acid.

4.4.1 N-Alkyl Phenylethylamine Metabolism

C. echinulata extensively metabolized (±)-N-(*n*-propyl)phenylethylamine (NPE), (±)-N,N-(di-*n*-propyl)phenylethylamine (DPE) and (±)-N-(isopropyl)phenylethylamine (IPE). The major metabolite in each instance was AcPHE with only trace quantities of PHE detected. However, recovery of AcPHE was low which would suggest that alternate metabolic

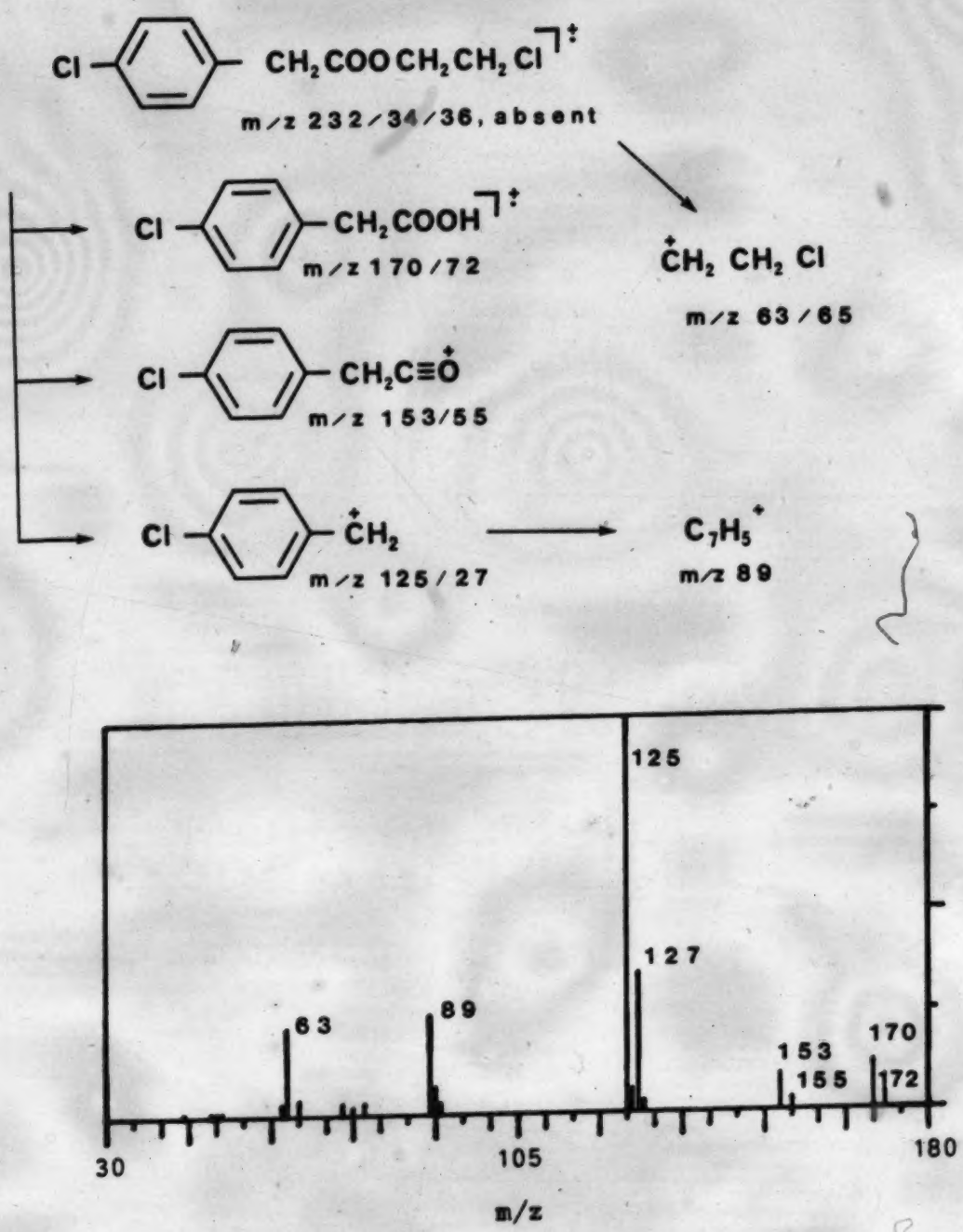
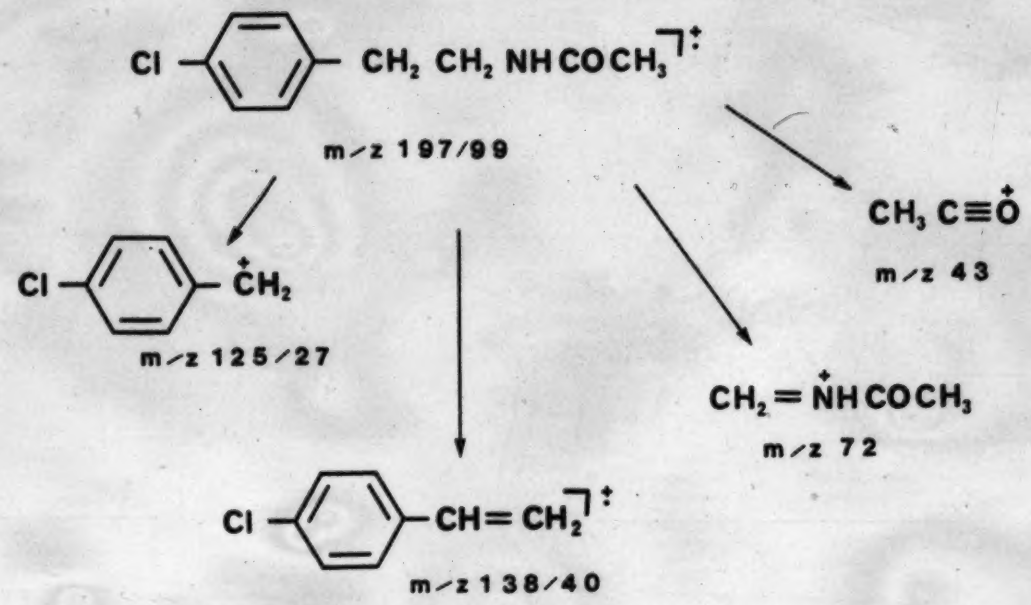


Figure 12 . Mass spectral fragmentation pattern for the chloroethanol derivative of *p*-chlorophenylacetic acid.



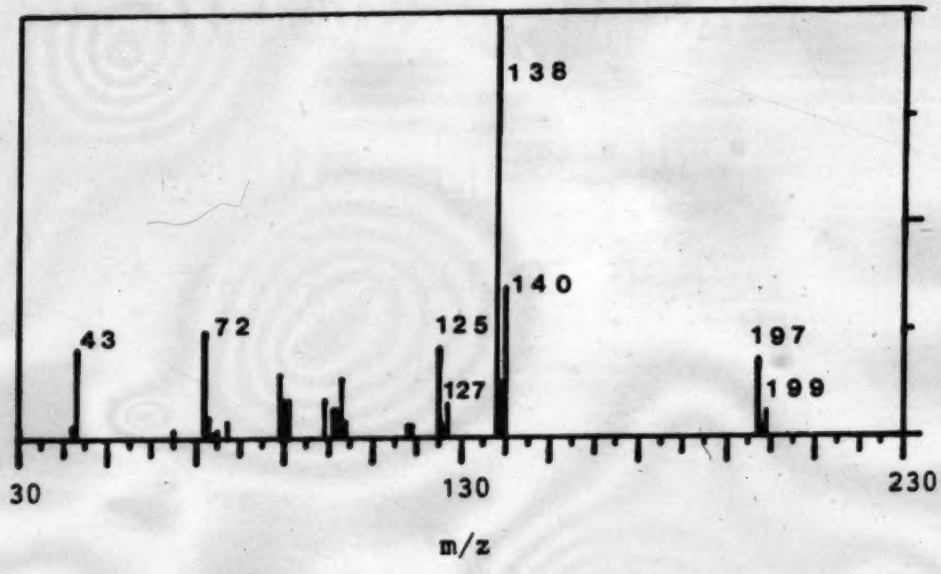


Figure 13 . Mass spectral fragmentation of N-acetyl-*p*-chlorophenethylamine, a metabolite of *p*-chlorophenylethylamine.

Table 11 . Metabolic patterns observed for the *M. smegmatis* biotransformation of phenylethylamines.

	Metabolic patterns[1]			
Substrate	OD	ND	NO	NAc
PHE	-	NA[2]	-	-
pCl-PHE	+++	-	-	-
NPE	-	-	-	-
DPE	-	-	-	-
IPE	-	-	-	-
AMPH	-	NA	+	+++
pCl-AMPH	-	NA	-	+++
NMA	-	+	+	++
NEA	-	+	++	++
NPA	-	-	+++	+++
IPA	-	-	+	-
NBA	-	+	++	++
PHT	-	NA	-	-
NMT	-	+++	-	-
NET	-	+++	++	-
NPT	-	+	++	-
IPT	-	-	-	-
NBT	-	++	++	-

[1](-) no metabolite observed, (+) trace metabolite, (++) minor metabolite, (+++) major metabolite. (OD) oxidative deamination, (ND) N-dealkylation, (NO) N-oxidation, (NAc) N-acetylation. [2] (NA), not applicable.

routes were present but not detectable. DPE was the only substrate still detectable after 168 h of incubation.

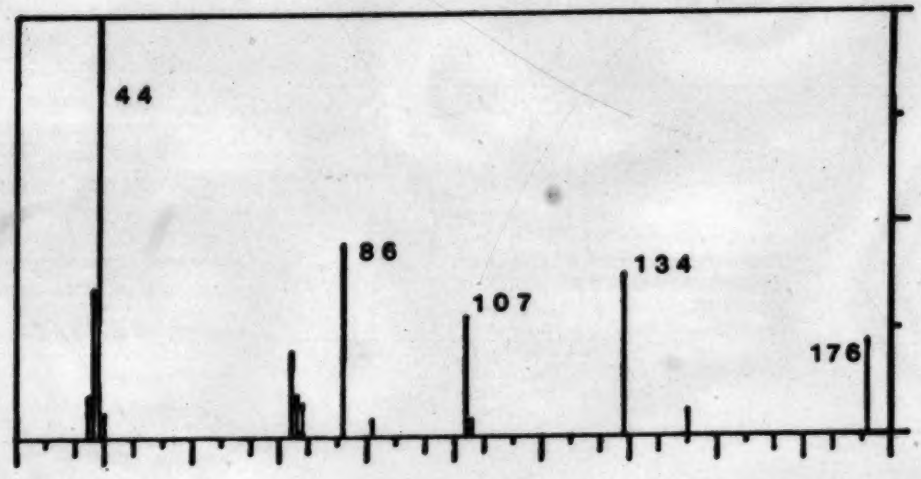
GLC examination of extracts from *M. smegmatis* incubation broths containing NPE, DPE or IPE revealed that these substrates were completely metabolized to undetectable products. DPE was observed at 96 h but was absent by 120 h.

Since the *p*-chloro analogs of NPE, DPE and IPE were not available, it was not possible to determine whether these derivatized phenylethylamines were metabolized by oxidative deamination in a manner analogous to CLE by these organisms.

4.5 Amphetamine Metabolism

The major biotransformation product of AMPH in the extracts of *C. echinulata* (Table 10) incubation broths was AcAMPH. Trace quantities of OXM and NOH-AMPH were also detected. Extraction of these broths under acidic conditions (Method B, section 3.4.1) and esterification with chloroethanol recovered only trace quantities of the chloroethyl derivative of benzoic acid. The amounts recovered though were not significantly different from the values observed in the control experiments. Derivatization of the incubation extract with acetic anhydride prior to extraction enhanced the recovery of *p*OH-AMPH (Fig. 14). A portion of this extract was treated with 7N ammonium hydroxide to selectively cleave the O-acetyl function. Examination of this reaction mixture revealed that the peak tentatively

(A) N,O-diacetyl-*p*-hydroxyamphetamine



(B) N-acetyl-*p*-hydroxyamphetamine

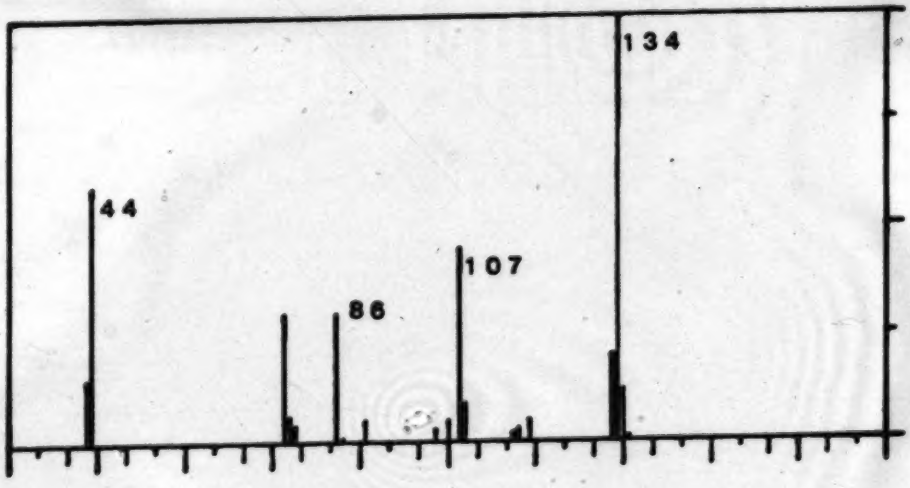


Figure 14 . Mass spectral fragmentation patterns for the N-acetylated derivatives of *p*-hydroxyamphetamine.

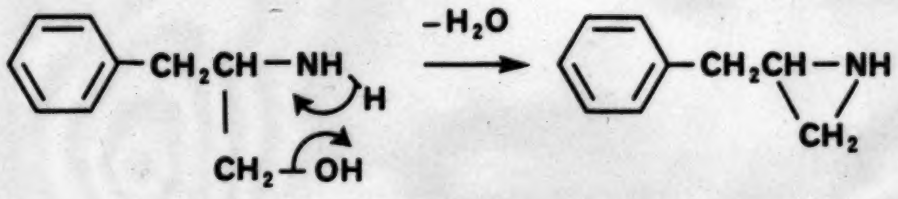
identified as the N,O-diacetate was absent, having been replaced by a new peak corresponding to the mono acetate derivative. Identification of *p*OH-AMPH was confirmed by comparison of the physical properties of authentic standard and its acetylated derivatives with those of the metabolite and the related diacetylated compound.

GLC examination of the acidic and alkaline extracts from a *M. smegmatis* incubation broth containing AMPH revealed the presence of a trace amount of OXM and a single major metabolite, AcAMPH (Table 11).

4.5.1 Iodoamphetamine

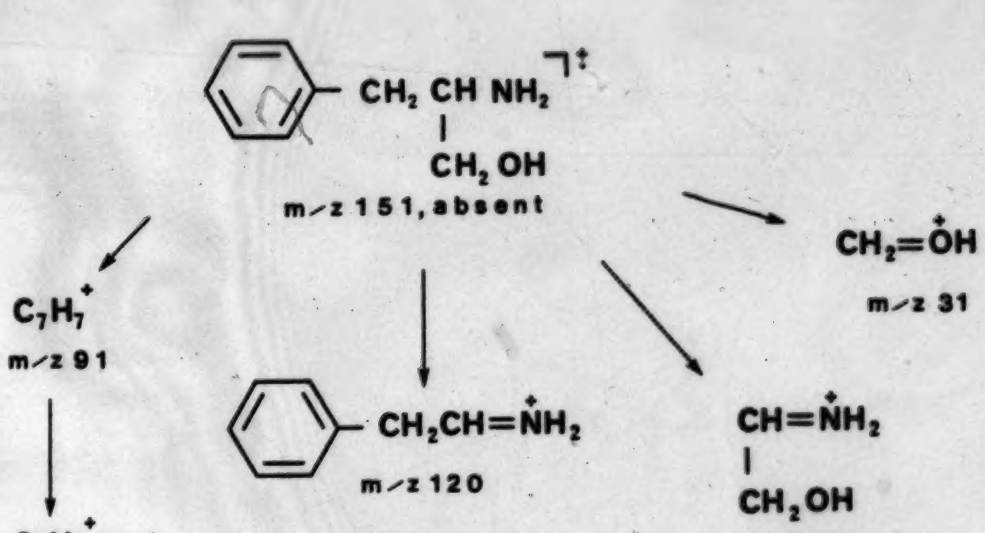
Halogenated amphetamines have a potential use as positive X-ray contrast materials (Herms and Taenzer 1975) and are being broadly employed as diagnostic, scanning and radiopharmaceutical agents (Counsell and Ice 1975). Little information is available though on the metabolic profile of these halogenated agents. 2-Amino-3-iodo-1-phenylpropane (IAM) was prepared for evaluation as a radiopharmaceutical (Coutts personal communication). When IAM was added to *M. smegmatis* incubation broths at a concentration of 7 mg/flask, the growth of this organism was severely restricted. The recovery of substrate from the incubation broth was less than that observed in media controls. Two additional compounds were present in both the control and incubation flask extracts. The alkaline extract (Method A, section 3.4.1) was analyzed by GLC-MS and the minor compound

was found to contain diagnostic fragmentation ions at m/z 60, m/z 91, and m/z 120. The production of these ions is consistent with those expected for 2-amino-3-phenylpropanol (APP) fragmentation (Fig. 15). The major product was found to contain diagnostic ions consistent with it being the aziridine (AZI, Fig. 16). This compound is formed by the loss of a molecule of water from IAM (Scheme 12)



Scheme 12

during the incubation and analytical procedure. The concentrations of APP and AZI increased with time and after several days the substrate was no longer detectable. *M. smegmatis* was unable to reproduce at a reasonable rate in the presence of IAM (7 mg). However, the cells were still physiologically active as determined by the absence of an endogenous medium component which was present in the substrate controls. When *M. smegmatis* was incubated with 1.7 mg of IAM the growth was normal. The inhibited microbial cells were harvested by vacuum filtration and washed with sterile media and resuspended in fresh medium without IAM. The cells were incubated in the usual manner and after 48 h the cell population was visibly increased.



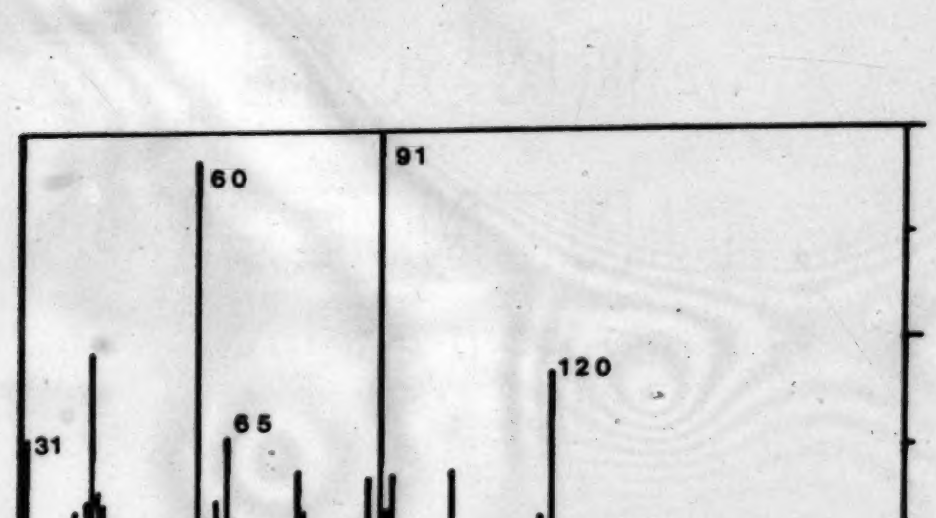
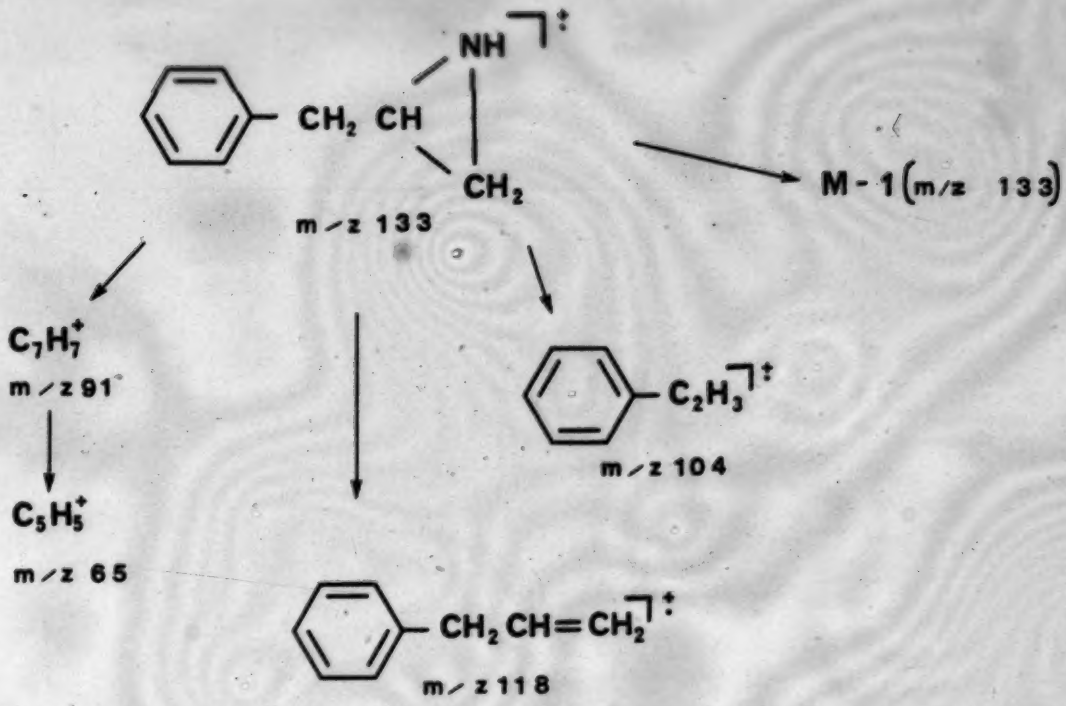


Figure 15 . The mass spectral fragmentation pattern of 2-amino-3-phenylpropanol, a decomposition product of 2-amino-3-iodo-1-phenylpropane.



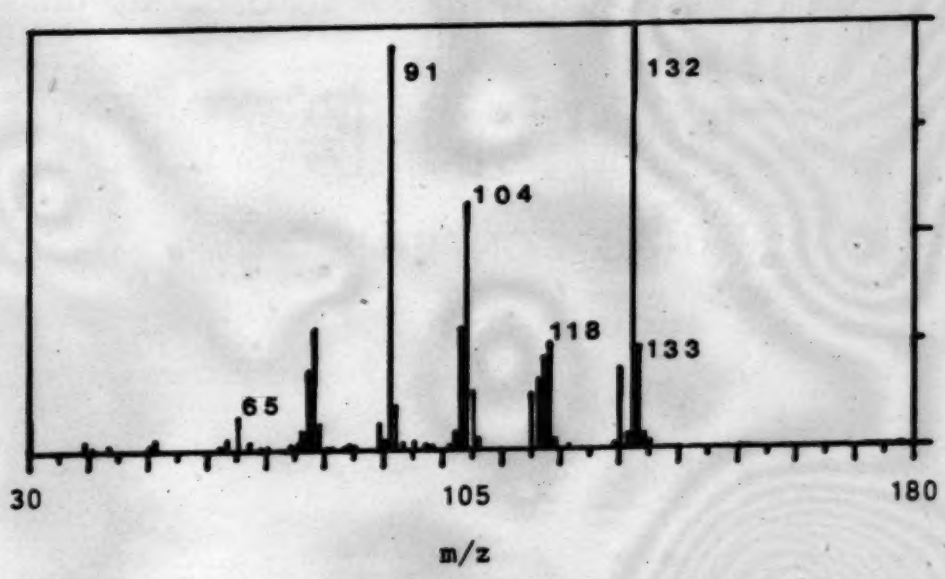


Figure 16 . Mass spectral fragmentation pattern of aziridine, a decomposition product of 2-amino-3-iodo-1-phenylpropane.

Addition of equal amounts (7 mg) of AMPH and IAM to 6 h stage II cultures of *M. smegmatis* resulted in restricted growth. GLC analysis of an extract from the broth after 48 h revealed that a portion of IAM had been transformed to APP and AZI. Amphetamine biotransformation was significantly altered in that the N-acetate was the only product recovered. The oxime (OXM), which is a normal product of this substrate was not detected.

In a second series of experiments IAM was added to test cultures of *M. smegmatis* at 0, 6 and 24 h of stage II growth without AMPH and at 6 h with AMPH. Controls and flasks containing AMPH showed good heavy growth after 72 h, as did the flask containing IAM added at 24 h. The addition of IAM, without amphetamine at 0 and 6 h was bacteriostatic.

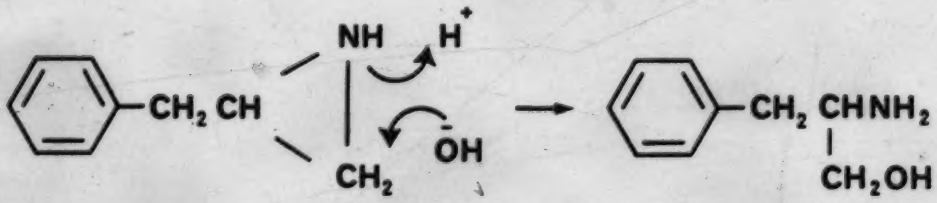
An overnight culture of *M. smegmatis* was suspended in nutrient agar and poured into petri plates and treated with four concentrations of IAM; 0.63, 1.25, 2.5 and 5 mg/mL in penicylinders. After 48 h the zones of inhibition were measured and the mean diameter was 0, 17, 27 and 37 mm, respectively. A 5X5 mm plug of agar-cell suspension was aseptically removed from the zones of inhibition and added to sterile nutrient broth. No growth was observed after an additional 48 h of incubation. It is concluded that IAM can exhibit either bacteriocidal or bacteriostatic control over *M. smegmatis* under different conditions.

The recovery of IAM from nutrient medium was considerably less than the recovery from phosphate buffer.

From this finding, it can be concluded that IAM can condense with endogenous media components or polymerize.

4.5.1.1 Aziridine

Aziridines have a wide range of biological properties and can act as anti-neoplastic agents (Brewster and Pinder 1972). On this basis, it was of interest to determine if the bacteriostatic effect observed in the IAM study was in fact due to AZI. The oxalate salt of AZI was added to stage II cultures of *M. smegmatis* (0.7 and 7.0 mg). The lower concentration was slightly inhibitory towards growth as it took four days for a culture to achieve the same biomass as a three day culture without IAM. The higher concentration was bacteriostatic, although the cells were still physiologically active as determined by the disappearance of the endogenous medium component in the incubation broth. Oxalic acid at the concentration present in the above samples was not inhibitory to *M. smegmatis*. APP was detected in both the control and metabolic extracts. It was presumably formed by hydration of the aziridine (Scheme 13).



Scheme 13

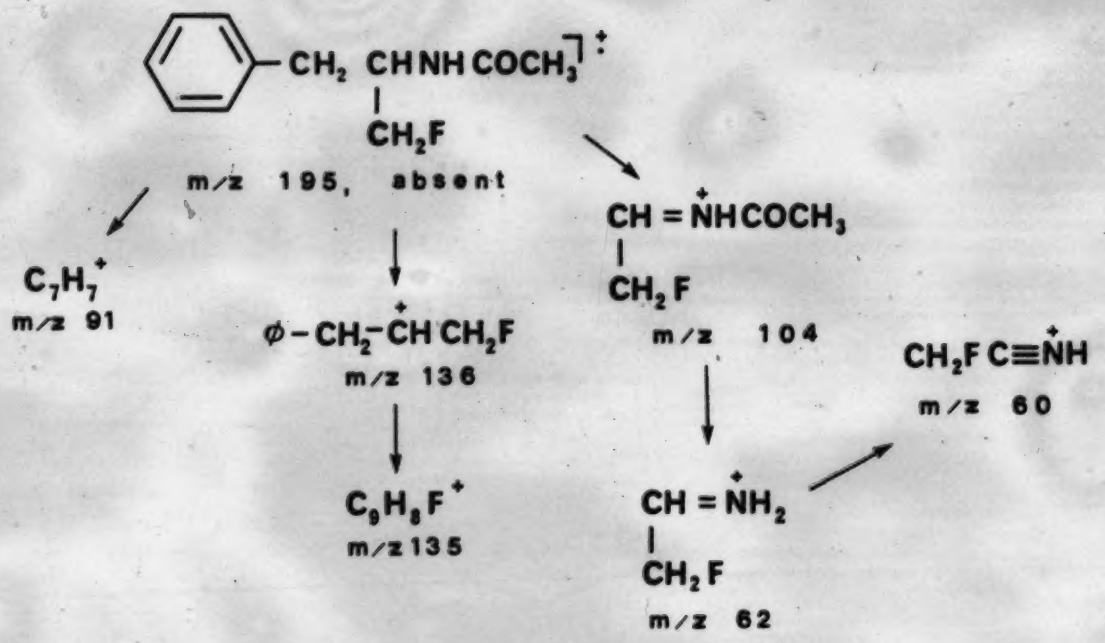
Occasionally a third compound was detected in the metabolic and control extracts. Its mass spectrum contained fragmentation ions at m/z 72 (65), m/z 91 (55), m/z 100 (100), m/z 117 (70), m/z 132 (20), and 162 (36). The identification of this compound has not been established.

Quantitative recovery of added AZI from the incubation medium was not possible. It is likely that the low recovery of AZI was due to its ability to act as an alkylating agent and react with cellular constituents (Brewster and Pinder 1972).

4.5.2 Fluoroamphetamine

Fluoroamphetamine HCl (2-amino-3-fluoro-1-phenyl-propane, FAM) was bacteriostatic at a concentration of 7.0 mg but not at 1.8 mg/flask when added to cultures of *M. smegmatis*. The culture was physiologically active and a long retention time metabolite was detected by GLC analysis in the alkaline extract from the 7 mg. Based on the known GLC properties and the diagnostic fragmentation pattern, the metabolite was identified as the N-acetyl conjugate of FAM, 2-acetylamino-3-fluoro-1-phenylpropane (AcFAM, Fig. 17) The structure was confirmed by comparison of its GLC/MS properties with the product of acetic anhydride treatment of FAM.

The metabolism of NPA was inhibited by the addition of 7 mg/flask FAM but not at a level of 1.8 mg.



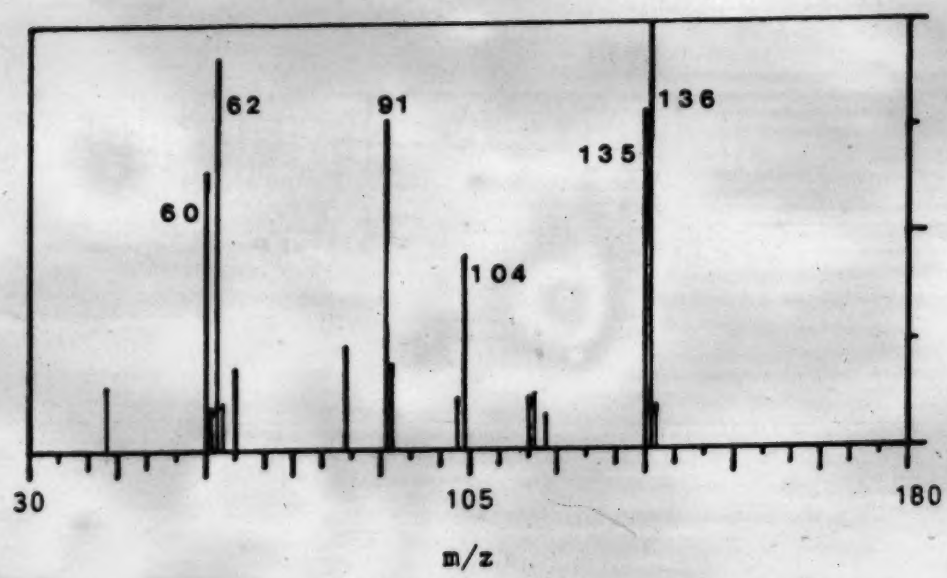


Figure 17 . The mass spectral fragmentation pattern of 2-acetylamino-3-fluoro-1-phenylpropane.

Metabolic profiles with *C. echinulata* and IAM, FAM and AZI were similar to those just described for *M. smegmatis*.

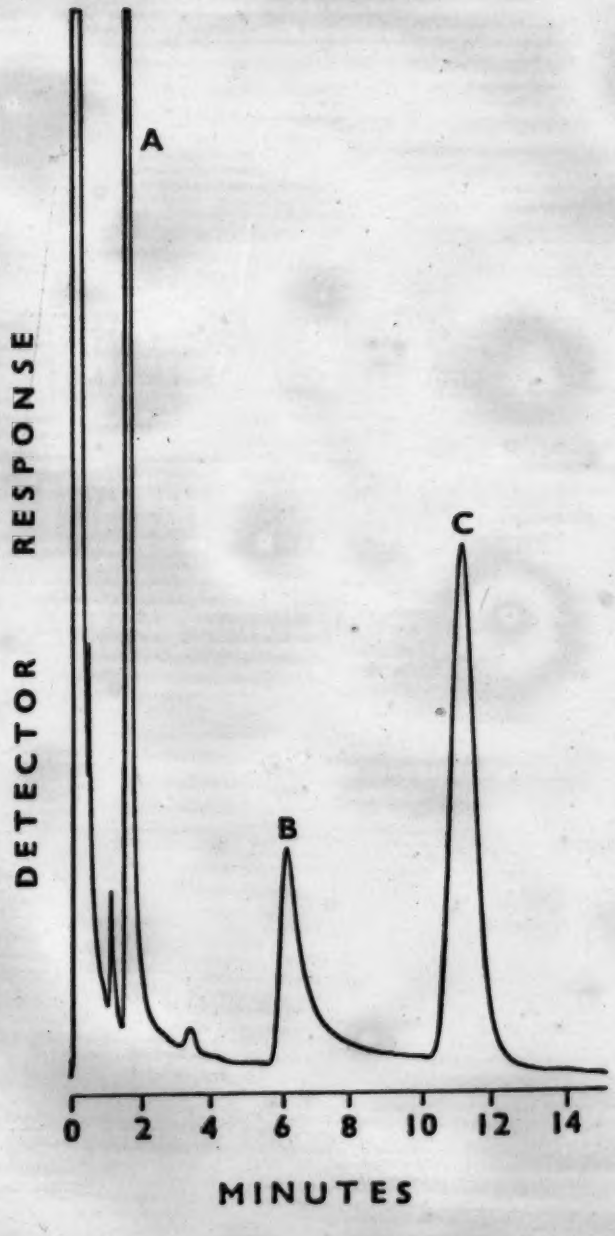
FAM may inhibit *M. smegmatis* by acting as an antimetabolite which can interfere directly or indirectly with various biosynthetic steps (Kollonish and Barosh 1976).

4.5.3 *p*-Chloroamphetamine

The incubation broth containing *M. smegmatis* and the substrate *p*-chloroamphetamine (CLA) was extracted (Method A, section 3.4.1) and analysed (Table 11). The results indicated that two new compounds identified as B and C were formed in substantial quantities (Fig. 18). Electron impact MS analysis of compounds B and C revealed that they had similar fragmentation patterns (Fig. 19 and 20 , respectively). The presence of diagnostic ions of m/z 125/127 and m/z 153/155 in both spectra indicated that both compounds had an intact 3-carbon side chain attached to a *p*-chlorophenyl group and that the metabolically added substituent was on the nitrogen. Prior to chemical ionization mass spectrometry the molecular weights of metabolites B and C were determined to be 237/239 and 239/241, respectively, by the addition of the diagnostic ions at m/z 112 or 114 to the m/z 125/127 fragment.

Chemical ionization MS analysis of each peak provided the molecular weights of B (mol. wt. 237/239) and C (mol. wt. 239/241). The MS pattern of compound B contained a major ion at m/z 194/196. Based on the molecular weight of this

Figure 18 . GLC trace of an *M. smegmatis* incubation broth containing (A) *p*-chloroamphetamine (CLA), (B) 4-chloro-*alpha*-methyl-N-(1-methylacetonylidine)benzeneethanamine (CBB), and (C)) 4-chloro-*alpha*-methyl-N-(1-methylacetonyl)benzeneethanamine (CBC). System B, 145°C.



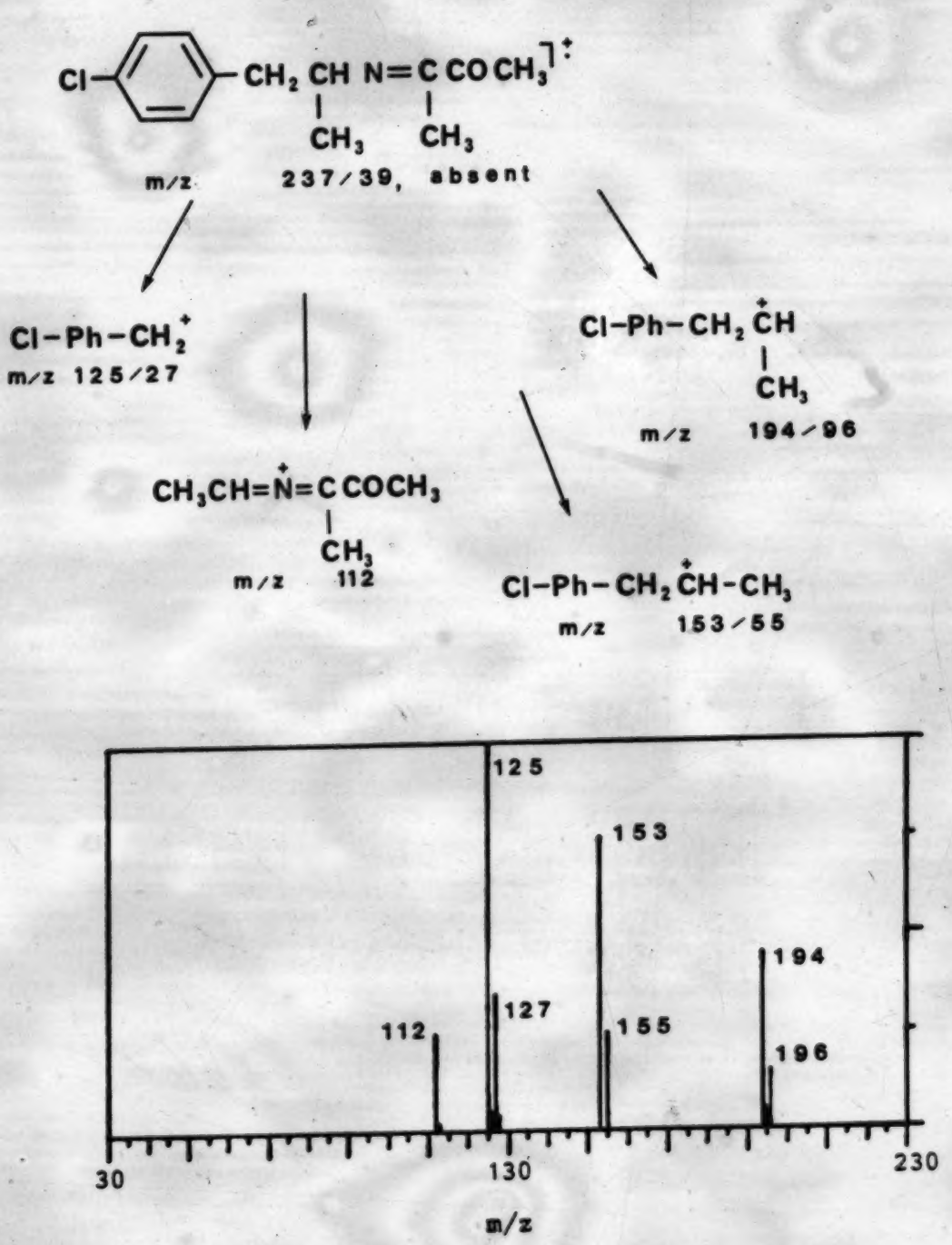
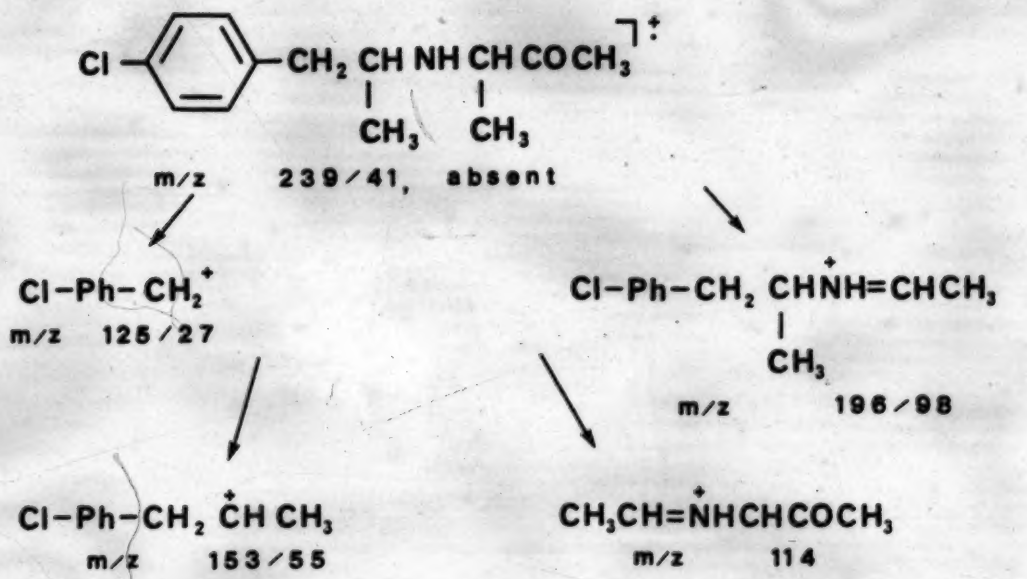


Figure 19 . Mass spectral data for the imine, 4-chloro-*alpha*-methyl-N-(1-methylacetonylidine)benzeneethanamine (CBB).



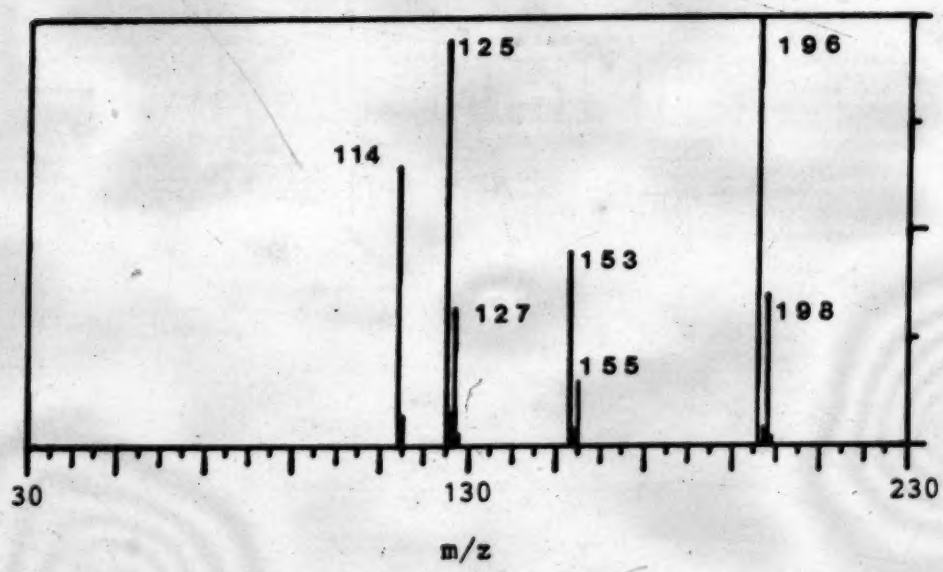
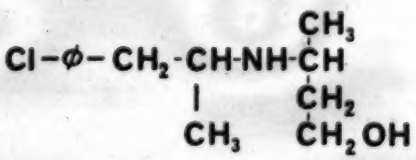


Figure 20 . The mass spectrum of 4-chloro-*alpha*-methyl-N-(1-methylacetonyl)benzeneethanamine (CBC), a acetoin derivative of *p*-chloroamphetamine.

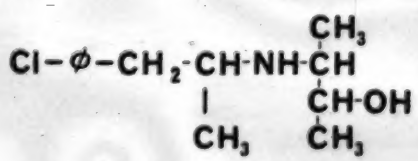
compound, it was evident that this ion represented the loss of 43 atomic mass units (ie. $COCH_3$, CH_3CHO, or C_3H_7) from the molecular ion. The ion, m/z 194/196, must therefore contain the full PCA molecule and a portion of the N-substituent. In the MS of C, the fragment ion m/z 194/196 in B was replaced by an ion at m/z 196/198, also presumed to be a M-43 fragmentation product. It was observed that C was slowly transformed to B upon storage at 4°C. These findings suggest that B was in fact a oxidation product of C.

If this conclusion was correct then treatment of a portion of the original extract with LAH or SBH should result in conversion of B to C (Brown and Krishnamurthy 1979; Zankowska-Jasinska and Holak 1976). In fact LAH reduction of this sample resulted in a marked decrease in the quantities of both B and C and a concomitant formation of a new compound, identified as D. The MS fragmentation pattern of D (Fig. 21) was similar to that of C.

These data can be readily interpreted if compound D has one of two structures [(a) or (b)]:



(a)



(b)

Both compounds were synthesized. The GLC/MS properties of (a) were different from those of compound D. The isomer (b),

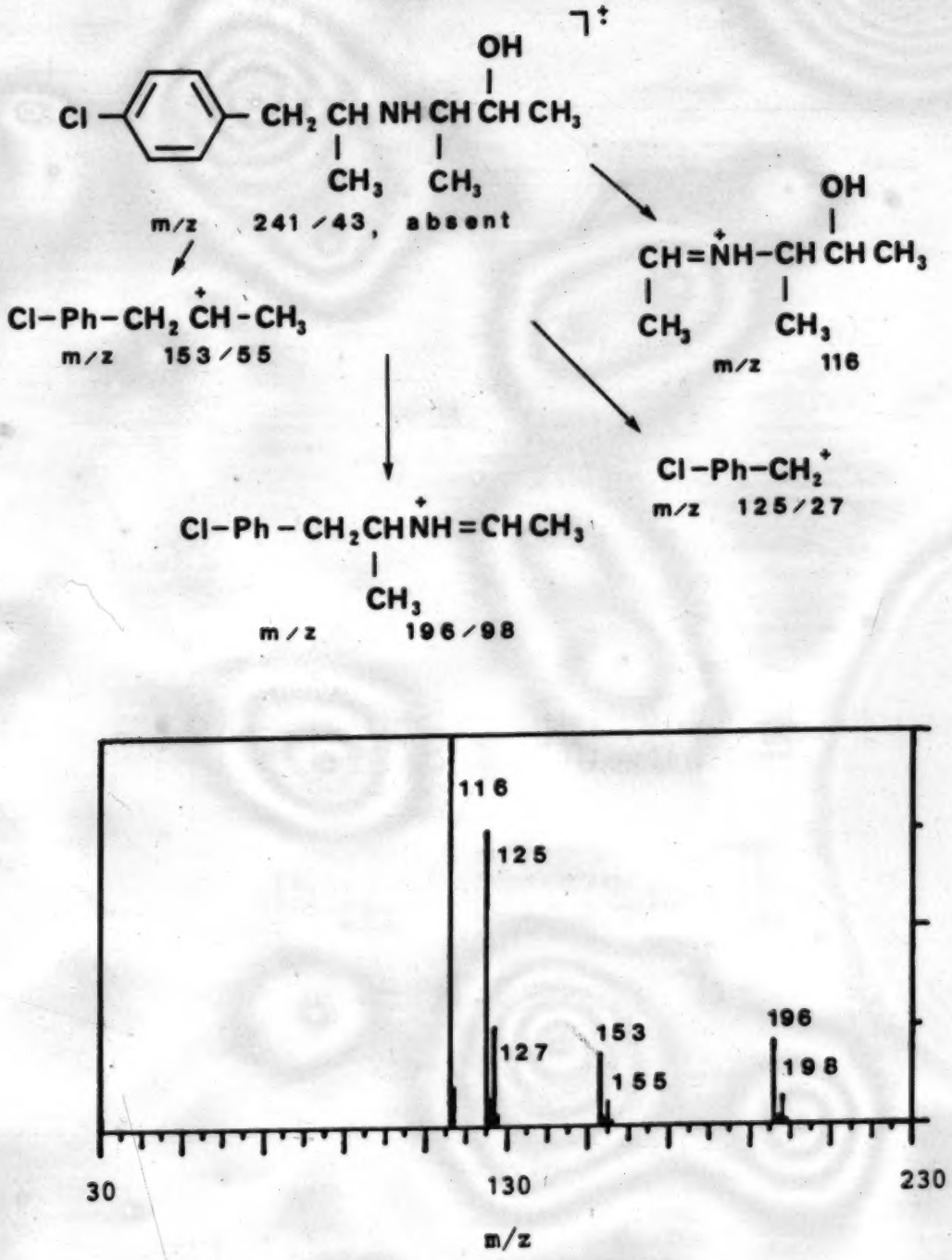


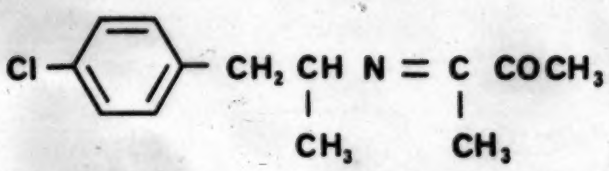
Figure 21 . The mass spectrum of 4-chloro-*alpha* methyl-N-(1-methyl-2-hydroxypropyl)benzeneethanamine (CBD), the LAH reduction product detected in the original extract.

had GLC/MS properties identical to those of D, which was unequivocally identified as 4-chloro-*alpha*-methyl-N-(1-methyl-2-hydroxypropyl)benzeneethanamine (CBD). It follows from this deduction and from mass spectral information that metabolite C is the ketone corresponding to structure (b) which has the structure shown in Fig. 22 and that metabolite B is the related imine (Fig. 22). Compounds B and C were then identified as 4-chloro-*alpha*-methyl-N-(1-methylacetonylidene)benzeneethanamine (CBB) and 4-chloro-*alpha*-methyl-N-(1-methylacetonyl)benzeneethanamine (CBC), respectively.

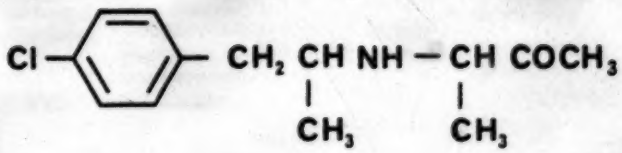
The intermediate product in the chemical preparation of CBD from PCA and acetoin was expected to be the imine, E (Fig. 22), but when this reaction was conducted in the absence of SBH, the imine was not detected by GLC. Instead, two intermediate products were formed which had GLC properties identical to those of CBB and CBC. By performing a time study of this reaction (Table 12), it was determined that CBC was the initially formed product which, on standing, underwent oxidation to CBB. The mechanism proposed for the formation of CBB and CBC is illustrated in Fig. 23 .

The formation of CBB and CBC by *M. smegmatis* can be explained if acetoin is present in the medium. CBB could also be formed if diacetyl was present. It was confirmed that the medium did not contain either of these compounds; CBB and CBC were not detected when PCA was incubated in sterile broth. When diacetyl was added to the broth, the

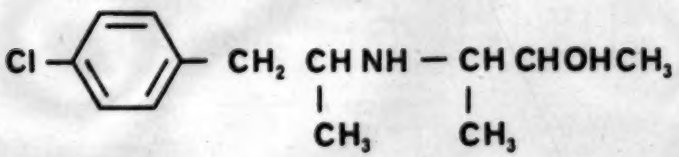
B. 4-chloro-*alpha*-methyl-N-(1-methylacetonylidine)benzeneethanamine



C. 4-chloro-*alpha*-methyl-N-(1-methylacetonyl)benzeneethanamine



D. 4-chloro-*alpha*-methyl-N-(1-methyl-2-hydroxypropyl)-benzeneethanamine



E.

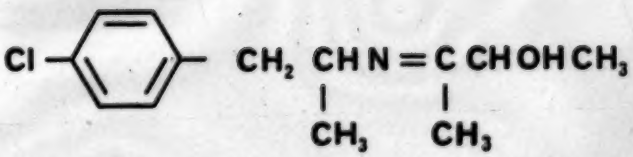


Figure 22 . Structures of *M. smegmatis* metabolites (B and C) of *p*-chloroamphetamine and related compounds (D and E).

Table 12 . Effect of time on the proportion of products obtained in the reaction of acetoin with *p*-chloroamphetamine.

	% yield of recoverable products[1]		
Time (h)	CLA	CBB	CBC
0	21.7	3.6	74.7
1	3.6	10.2	86.2
9	-	19.3	80.7
22	-	34.0	66.0
30	-	33.0	67.0
70	-	41.0	59.0
166	-	49.3	50.7
336	-	83.2	16.8

[1] Based on relative peak areas.

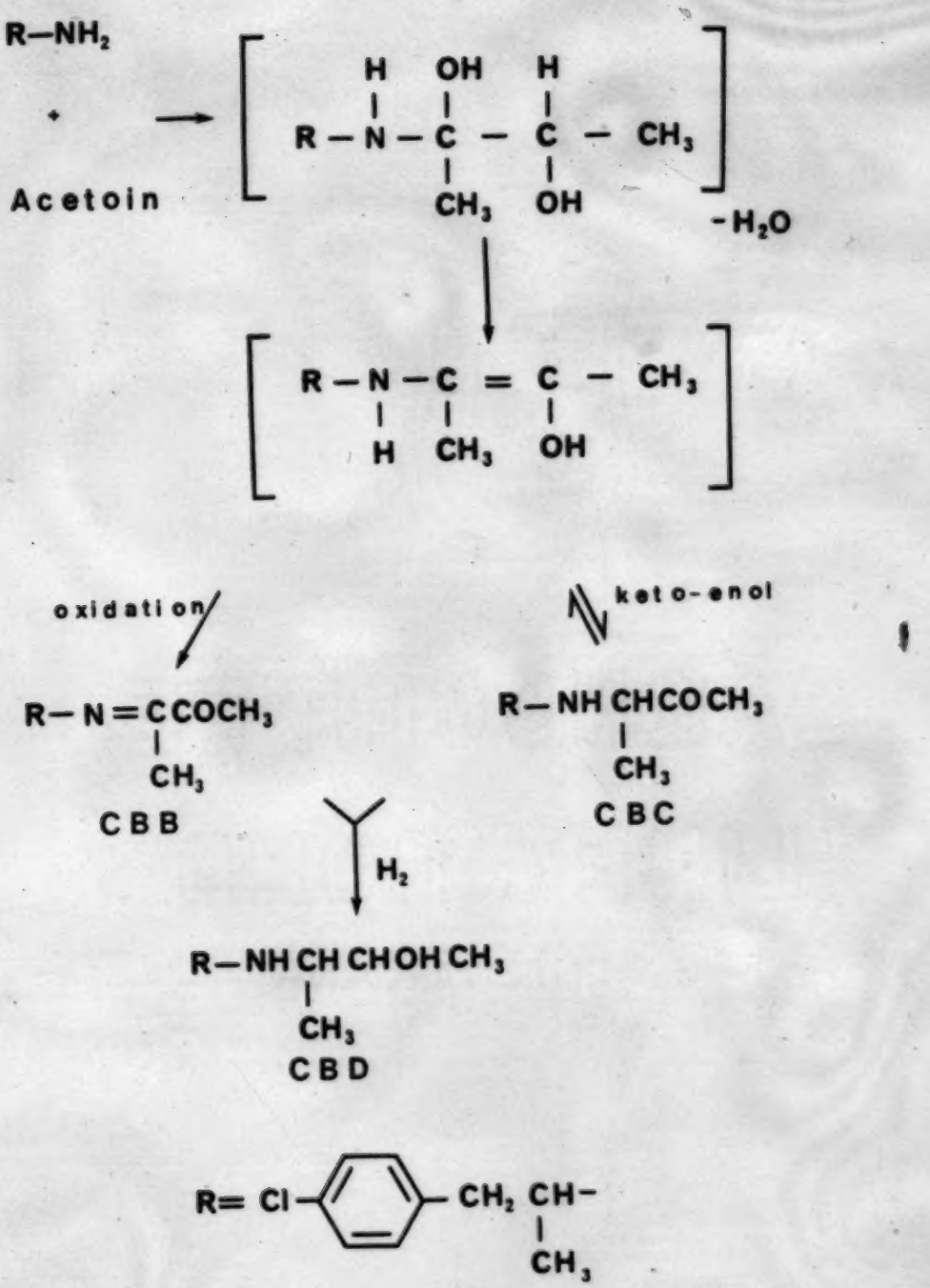
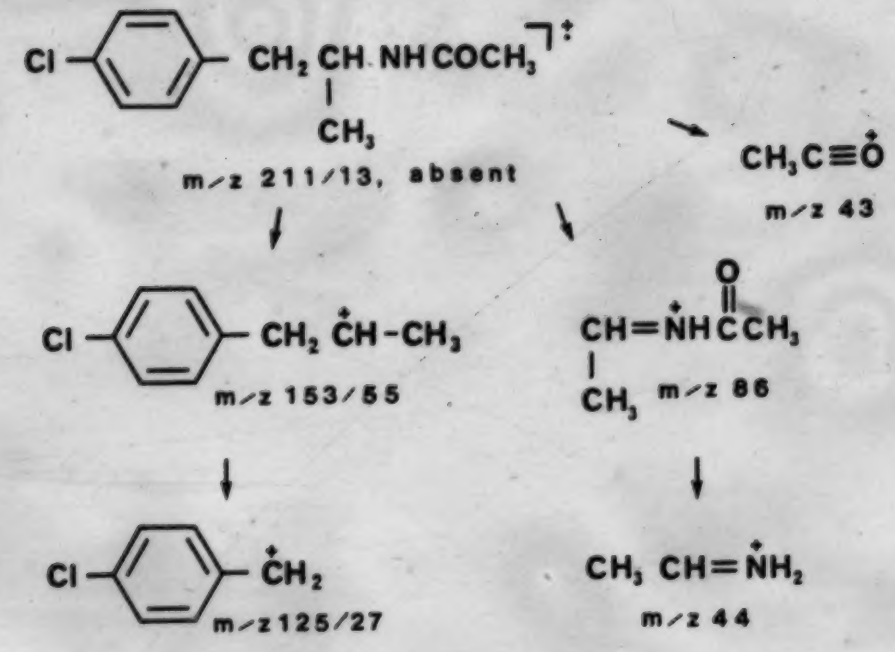


Figure 23 . The proposed mechanism for the formation of 4-chloro-*alpha*-methyl-N-(1-methylacetonylidine)benzeneethanamine (CBB), 4-chloro-*alpha*-methyl-N-(1-methylacetonyl)benzeneethanamine (CBC), and 4-chloro-*alpha*-methyl-N-(1-methyl-2-hydroxypropyl)benzeneethanamine (CBD).

imine CBB was rapidly produced as the single product. When acetoin was added to fresh broth, both compounds were obtained. These results indicate that the acetoin (and perhaps diacetyl) utilized in the formation of CBB and CBC was produced by *M. smegmatis*.

It is noteworthy that CBB and CBC, which were detected as early as 18 h after commencement of stage II growth, were the only N-derivatized products obtained in the present study. No N-acetylated PCA was detected. This is a surprising observation as earlier studies found that *M. smegmatis* possessed the ability to N-acetylate amphetamine. Further studies resolved this apparent paradox. *M. smegmatis* was repeatedly subcultured at day 5 of stage II and after 5-7 subcultures the organism no longer produced acetoin. Further studies did not reveal a reversion to acetoin production. GLC analysis of the extracts of later cultures revealed that they were devoid of CBB and CBC, but contained a new metabolite. This metabolite was identified as N-acetyl-*p*-chloroamphetamine (AcCLA, Fig. 24) on the basis of its GLC-MS properties, compared with those of an authentic standard.

Previous mycobacterial metabolism and taxonomic studies have not identified acetoin formation as a metabolic characteristic of *M. smegmatis* (Krulwich and Pelliccione 1979; Ramakrishnan *et al*. 1972; Ratledge 1976; Runyon 1974; Tsukamura and Mizuno 1968).



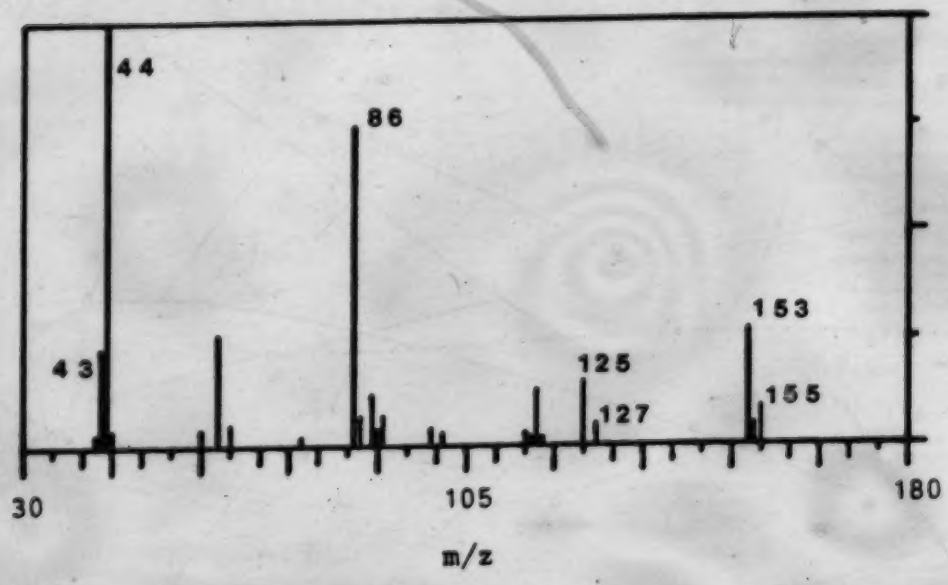


Figure 24 . N-acetyl-*p*-chloroamphetamine mass spectral fragmentation pattern.

Numerous organisms are listed in Bergeys Manual of Determinative Bacteriology (1974) as acetoin producers. It was of interest to determine if this conjugation could be mediated by other acetoin-producing organisms. Extracts of incubation broths containing either *Serratia marcescens* or *Klebsiella pneumoniae* and CLA were analyzed by GLC and found to be virtually identical to *M. smegmatis* extracts. Both organisms produced CBB and CBC.

4.5.3.1 Acetoin Derivatives of Amphetamine

In earlier studies dealing with the biotransformation of amphetamine by *M. smegmatis* it was indicated that the N-acetylation product was the major metabolite. Two other compounds were occasionally detected but always in trace amounts. They remained unidentified initially but in view of the studies described in section 4.5.3, it was now of interest to determine if these unidentified products were also acetoin derived conjugates. Amphetamine was added to a regenerated lyophilized culture of *M. smegmatis*. GLC analysis of the extracts of this broth showed the presence of two new compounds, identified as F and G. The GLC retention times of these compounds were identical to those of the unknown trace products. EI-MS revealed that they had fragmentation patterns similar to the corresponding CLA derivatives. SBH reduction of a portion of the original extract containing compounds F and G resulted in a marked decrease in the GLC peak areas of both compounds and a

concomitant formation of a new compound, H. Comparison of GLC and GLC-MS properties of authentic standards confirmed the identity of F, G and H as *alpha*-methyl-N-(1-methylacetonylidene)benzeneethanamine (ABF), *alpha*-methyl-N-(-1-methylacetonyl)benzeneethanamine (ABG) and *alpha*-methyl-N-(1-methyl-2-hydroxypropyl)benzeneethanamine (ABH, Fig. 25). AcAMPH was produced only after several subcultures as described above, by which time the acetoin-derived conjugates were no longer formed.

The formation of acetoin and diacetyl conjugates of drugs and xenobiotics has not been previously observed in mammalian or microbial systems.

4.5.3.2 Application of Diacetyl Assay

During the above studies with acetoin and diacetyl conjugates it became evident that there was no single simple method for the specific and accurate determination of acetoin and diacetyl in commercially important products and microbial incubation broths. Currently, these compounds can be detected by spectrophotometric (West 1978) and gas-liquid chromatographic (Lee and Drucker 1975) methods but both methods are relatively cumbersome and inaccurate due to the volatility and poor solvent solubility of acetoin and diacetyl. The problem of volatility and solubility was overcome by treating a solution containing these compounds with *o*-phenylenediamine to form 2,3-dimethylquinoxaline. Baseline separation of endogenous medium components from the

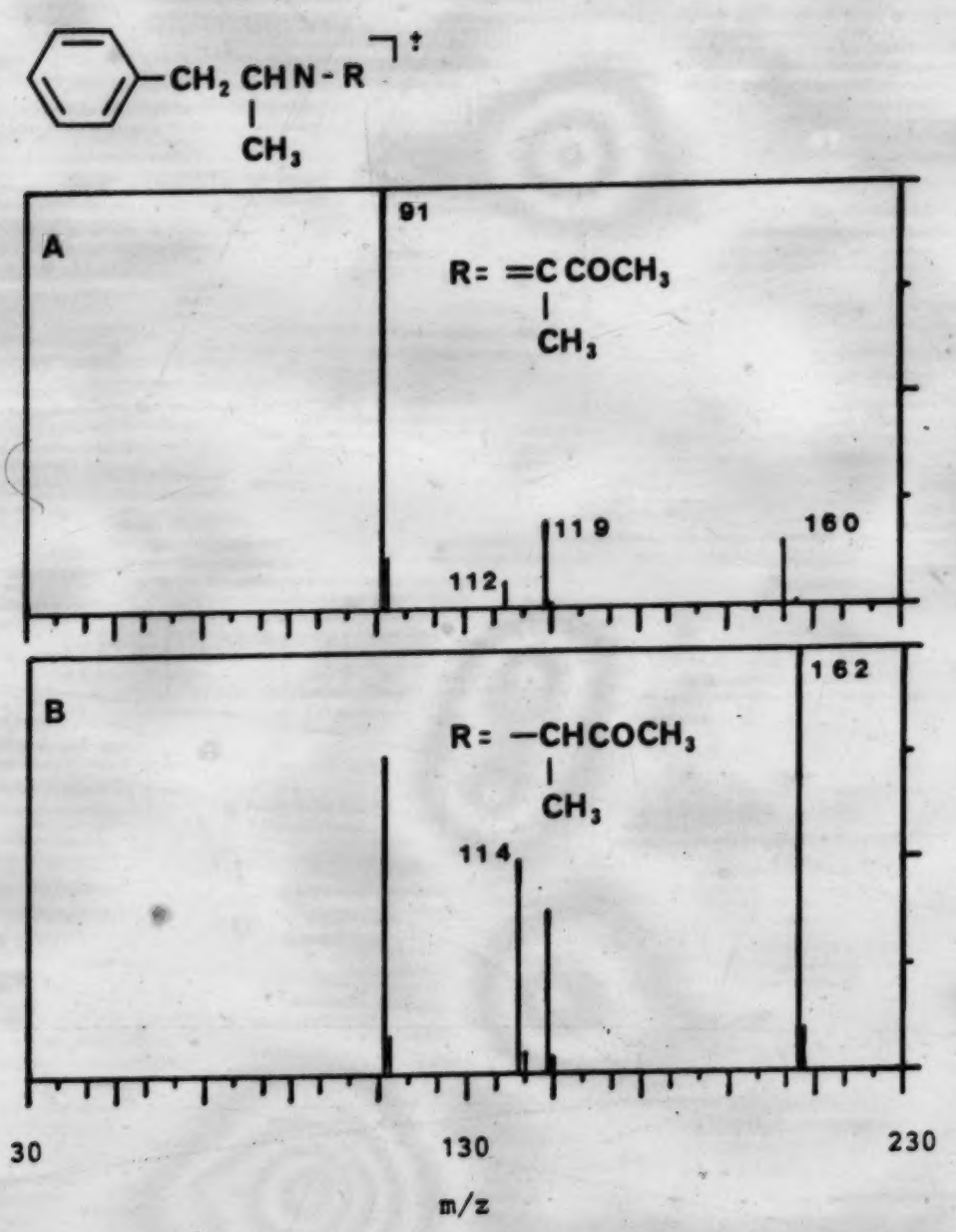


Figure 25 . The mass spectrum of the products obtained from the *M. smegmatis* metabolic extracts containing amphetamine. (A) *alpha*-methyl-N-(1-methylacetonylidine)benzeneethanamine (ABF) and (B) *alpha*-methyl-N-(1-methylacetonyl)-benzeneethanamine (ABG).

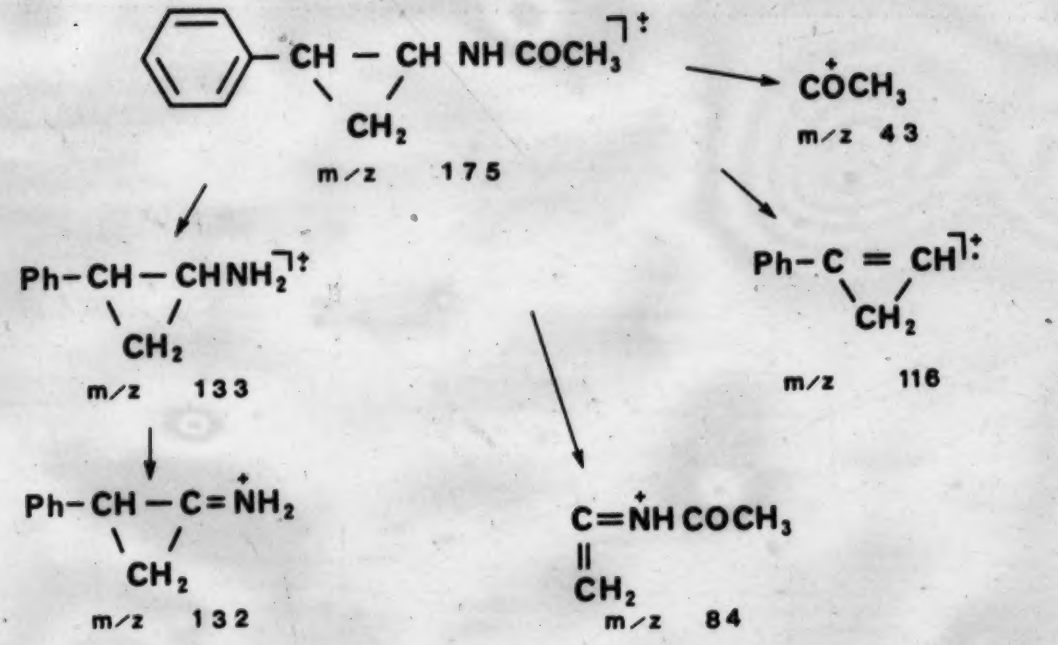
quinoxaline was achieved using column B, 175° (section 3.4.5).

Analysis of an extract from a 5 mL aliquot of Alta 3.9 (Carling O'Keefe Breweries) detected trace amounts of diacetyl. This assay provides a rapid, economical procedure for the accurate detection of acetoin and diacetyl in microbial incubation broths and malt products.

4.5.4 Tranylcypromine

Tranylcypromine (2-phenylcyclopropanamine, TCA) is a MAOI used as an antidepressant. The metabolic profile of this amine is poorly documented; only one study has been reported. Alleva (1963) found that in rat, TCA was rapidly metabolized to hippuric and benzoic acids with traces of four additional products. In aqueous solution TCA decomposed to ammonia and hydrocinnamaldehyde which was further converted to benzoic, and hydrocinnamic acid (Alleva 1963).

GLC analysis of the alkaline extract (Method A; section 3.4.1) from *M. smegmatis* cultures containing TCA revealed that this substrate was completely metabolized and only one product was recoverable under these conditions. The mass spectrum of this compound contained characteristic ions of TCA (Belanger 1979) and of acetate (m/z 43) which were consistent with it being N-acetyl-2-phenylcyclopropanamine (AcTCA, Fig. 26). The structure was readily confirmed by allowing a few crystals of TCA to react with acetic anhydride. The resulting product was found to have physical



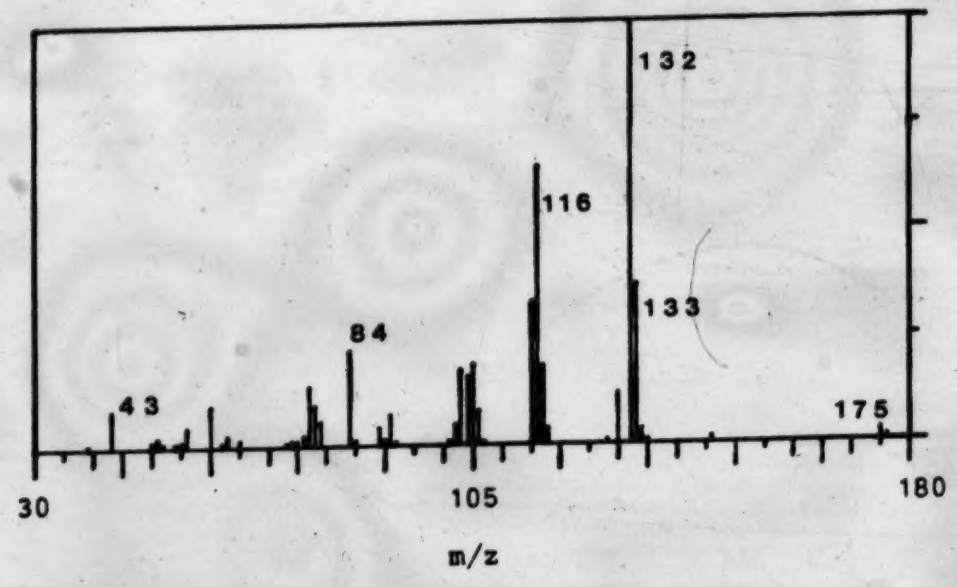
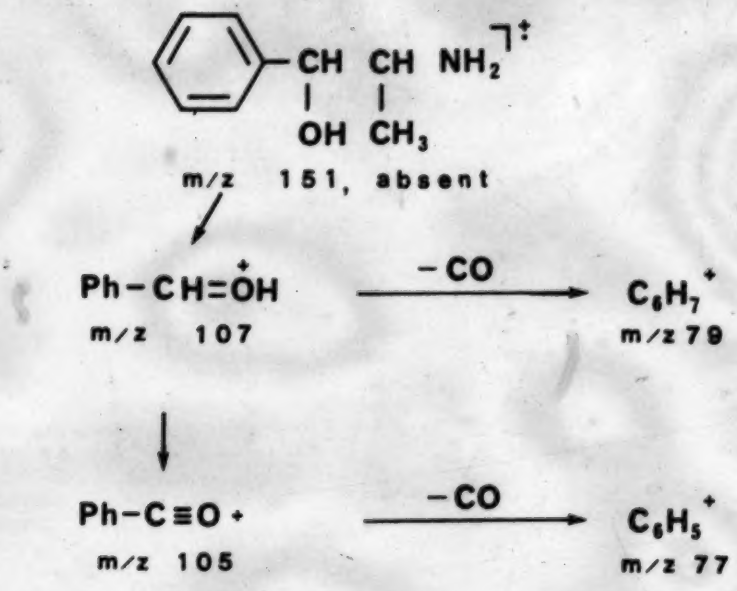


Figure 26 . The mass spectrum of N-acetyl-2-phenylcyclopropanamine and its fragmentation pattern.

characteristics identical to those of this metabolite.

AcTCA was also detected in the acidic extract of this metabolic reaction (Method B; section 3.4.1). Both extracts were otherwise not significantly different in content from those of the controls.

C. echinulata mediated the formation of a single TCA product, AcTCA. The substrate was not detected in extracts after 96 hours of stage II incubation. The N-acetylation product, AcTCA, was the only recoverable product when this organism was grown in Czapek Dox medium with dextrose and medium supplemented with dextrose and either malt extract (1 g/L) or yeast extract (1 g/L). It is noteworthy that both TCA and AcTCA were recovered when this organism was incubated in a medium consisting of 4% trypicase soy broth in 0.05M phosphate buffer, pH 7.4 and dextrose; in addition, a second metabolite was observed in the GLC trace. The MS fragmentation pattern of this metabolite did not contain characteristic TCA ions (Fig. 27). The base peak of this metabolite was the fragmentation ion m/z 107 which was highly diagnostic of a ring or β-hydroxylated metabolite. *p*OH-AMPH has a similar fragmentation pattern to that of the metabolite but the GLC retention time of *p*OH-AMPH was different. The GLC/MS properties of the β-hydroxylated product, norephedrine, were similar to those of this metabolite. This metabolite, however, was not detected in any subsequent study.



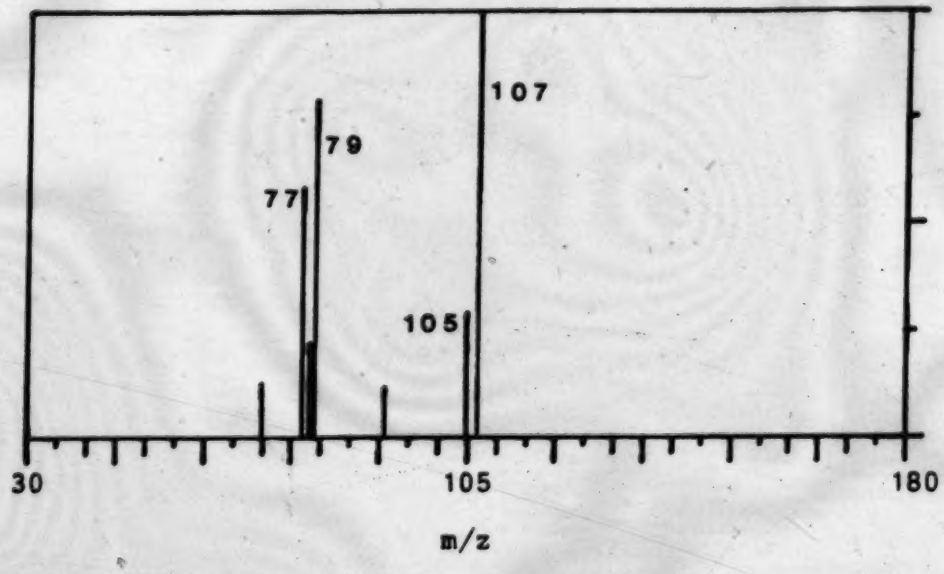
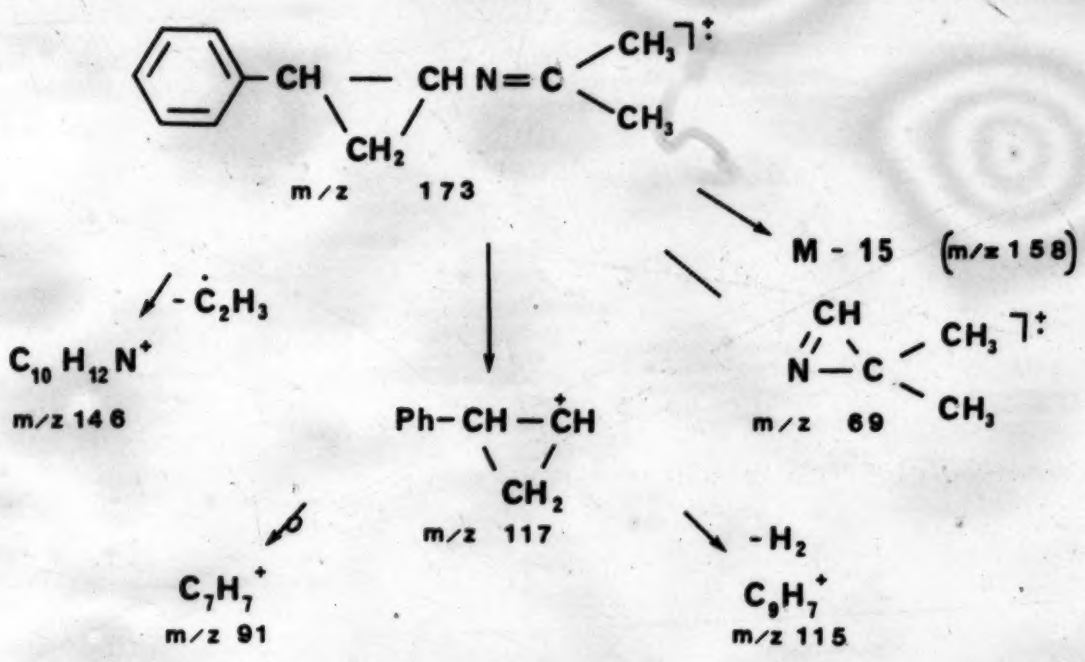


Figure 27 . The mass spectral properties of norephedrine, a possible metabolite of 2-phenylcyclopropanamine.

When the incubation temperature was altered from 27°C to 37°C the metabolic profile changed. Cultures of *C. echinulata* not supplemented with dextrose transformed TCA quantitatively to AcTCA. However, if dextrose was present in the incubation broth, AcTCA was not detected and three new peaks were observed in the GLC trace of the extract (Method A; section 3.4.1). The major metabolite had diagnostic fragmentation ions consistent with a N-substituted metabolite. The mass spectrum was found to have ions consistent with those expected from an acetone conjugate of the free amine (Beckett *et al.* 1979) (Fig. 28). The identity of this metabolite was confirmed as N-(acetonyl-idene)-2-phenylcyclopropanamine by comparing its GLC/MS properties with those of an authentic sample of this compound obtained by allowing acetone to react with the free base of TCA. The remaining two metabolites were not identified. Neither compound was the diacetyl conjugate of TCA which had physical properties different from those of either unidentified metabolite.

It was confirmed that the medium did not contain acetone; the GLC trace of an extract of the substrate control was devoid of this acetone conjugate. To ensure that the conjugate was not a result of acetone in the solvents or extraction tubes, the sample was re-extracted using freshly distilled solvents and oven dried glassware. The GLC profile of the extracted product was identical to the one previously observed. This confirmed that *C. echinulata* was responsible



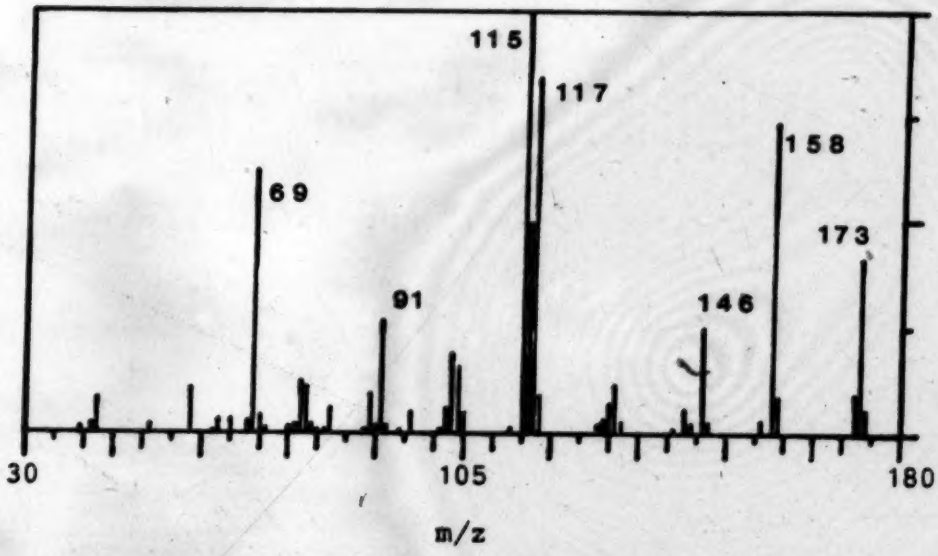


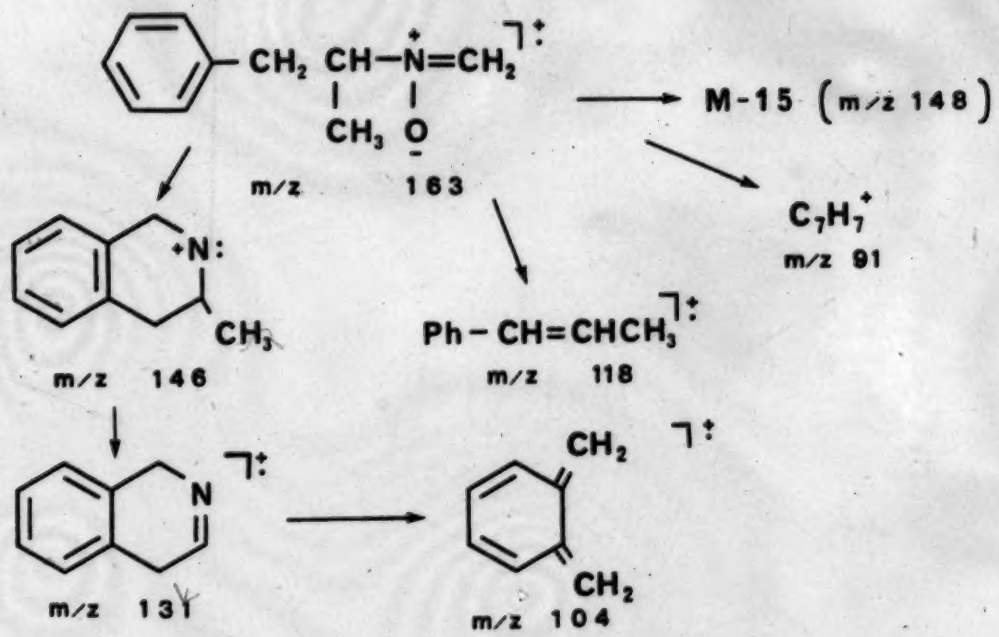
Figure 28 . Mass spectral fragmentation pattern of the acetone conjugate of 2-phenylcyclopropanamine, N-(acetonylidine)-2-phenylcyclopropanamine.

for the production of acetone and the resulting conjugate.

4.6 N-Alkylamphetamines

The original extracts and LAH reduced extracts from *M. smegmatis* cultures containing AMPH, NMA, (±)-N-(ethyl)-amphetamine (NEA), (±)-N-(isopropyl)amphetamine (IPA) and (±)-N-(*n*-butyl)amphetamine (NBA) were each examined by GLC and GLC-MS. Results are summarized in Table 11. The major metabolite observed in the AMPH extract was the N-acetyl conjugate AcAMPH and trace amounts of OXM. Trace quantities of AMPH and minor amounts of AcAMPH were isolated from the cultures containing NMA, NEA and NBA, but neither of these compounds was formed from IPA. However, all four N-alkylated substrates were metabolized to the corresponding nitrones *alpha*-methyl-N-(methylene)benzeneethanamine N-oxide (MOX) (Fig. 29), *alpha*-methyl-N-(ethylidene)benzeneethanamine N-oxide (EOX) (Fig. 30), *alpha*-methyl-N-(isopropylidene)-benzeneethanamine N-oxide (IOX) (Fig. 31), and *alpha*-methyl-N-(*n*-butylidene)benzeneethanamine N-oxide (BOX), respectively (Fig. 32).

The identification of MOX and EOX was readily confirmed by comparison of their GLC/MS physical properties with those of authentic standards and reported mass spectra (Coutts *et al*. 1978; Coutts and Kovach 1977; Beckett and Haya 1977; Coutts and Jones 1978). The presence of abundant ions of m/z 91, m/z 116 and m/z 117 in the spectra of IOX and BOX are



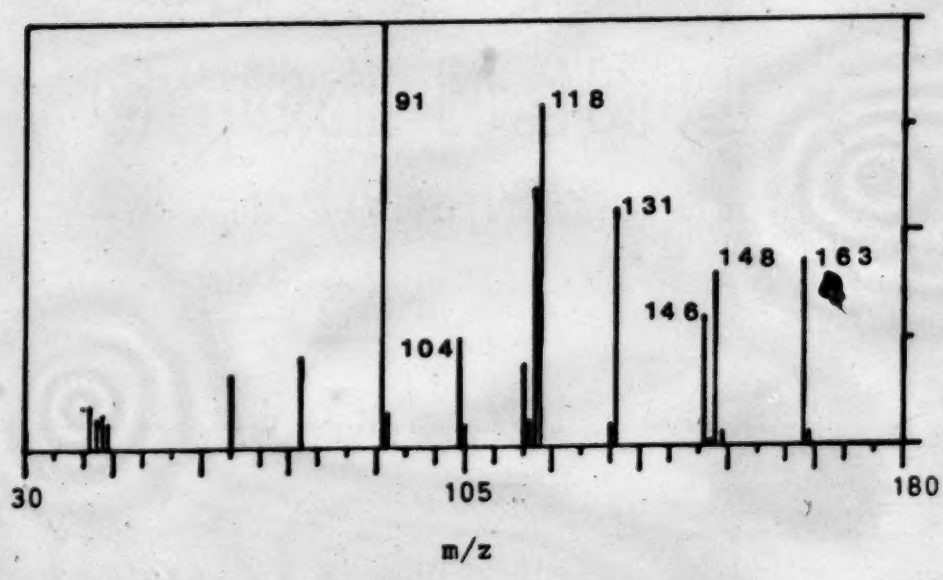
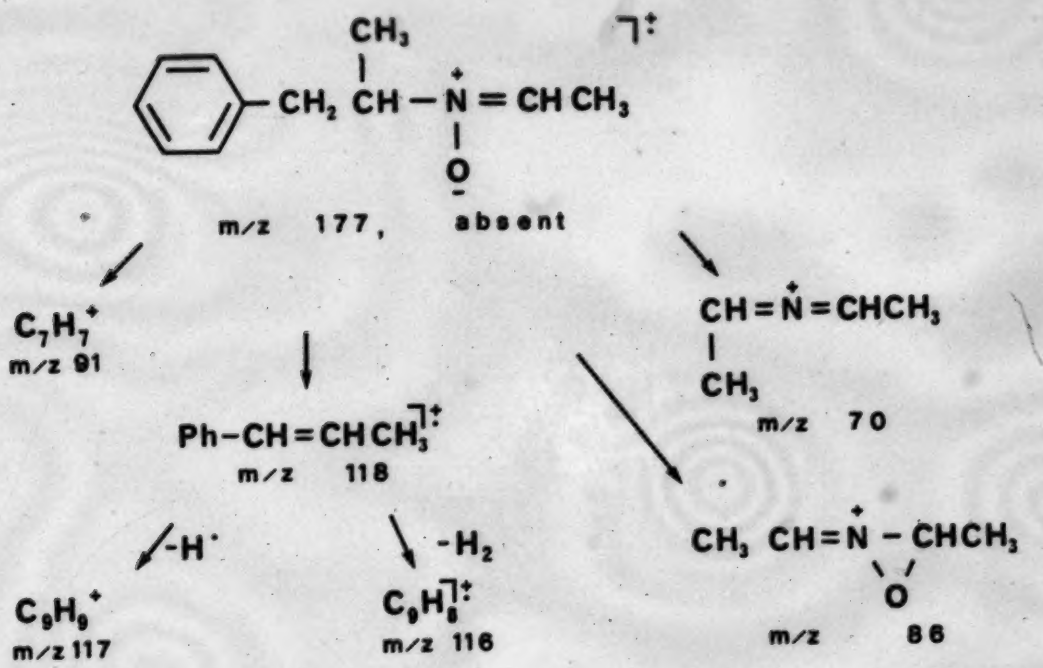


Figure 29 . Mass spectra of *alpha*-methyl-N-(methylene)benzeneethanamine N-oxide (MOX).



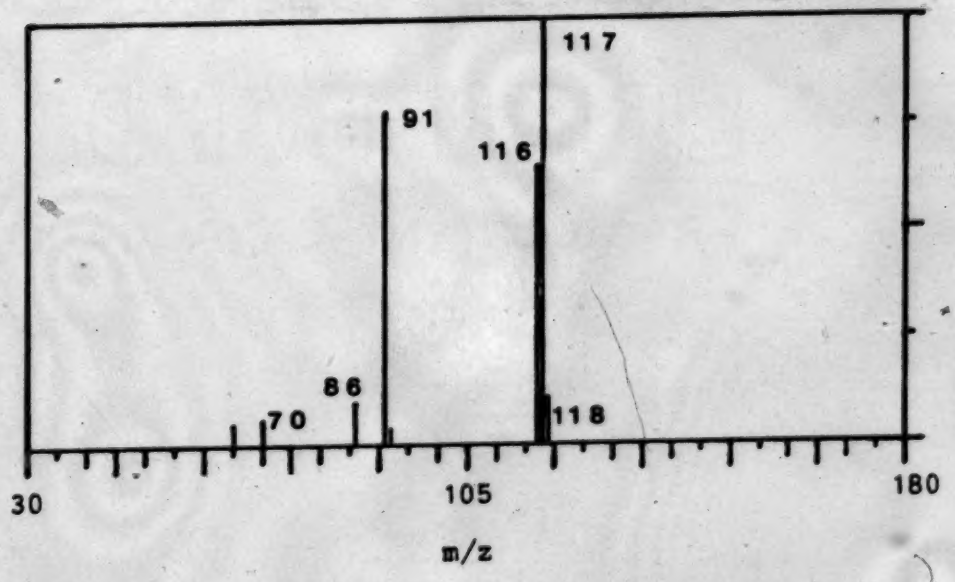
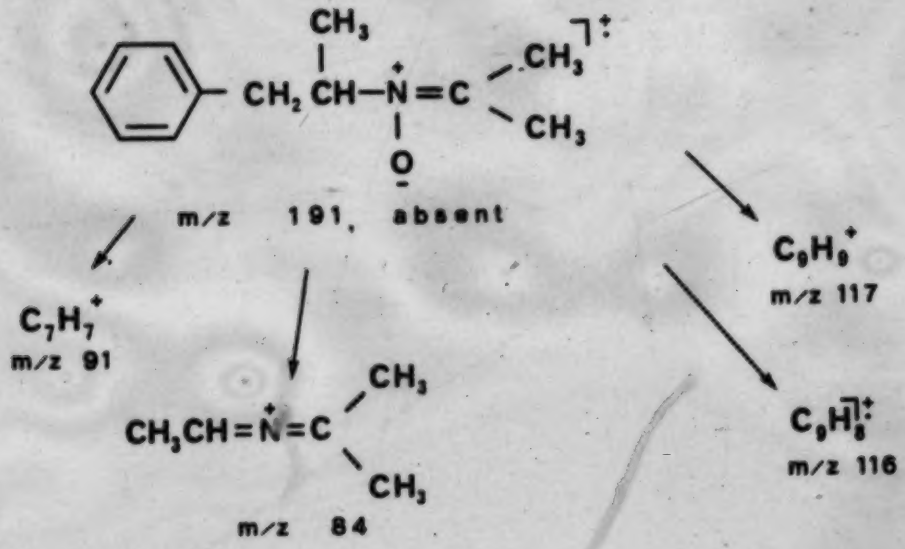


Figure 30 . Mass spectral fragmentation pattern of *alpha*-methyl-N-(ethylidene)benzeneethanamine N-oxide (EOX).



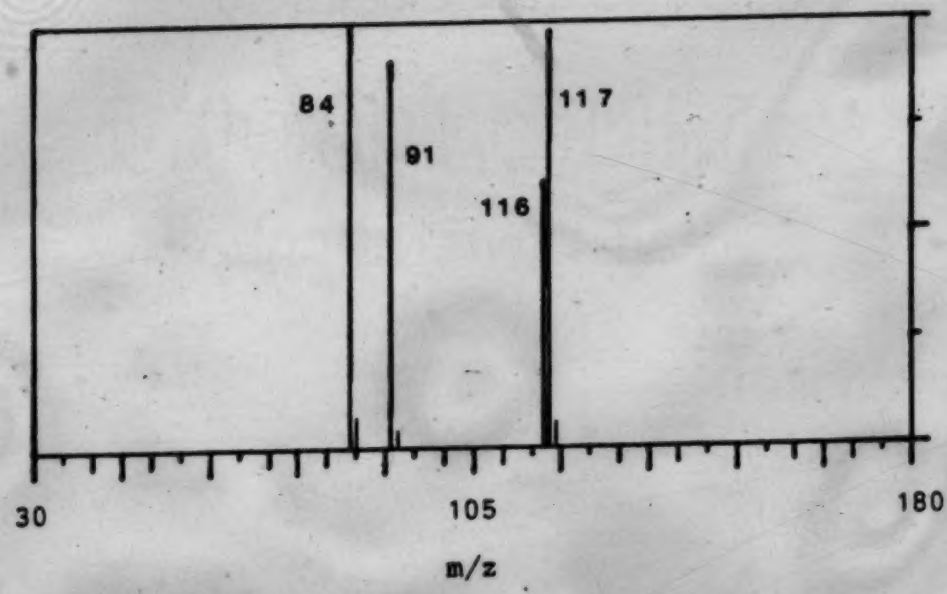
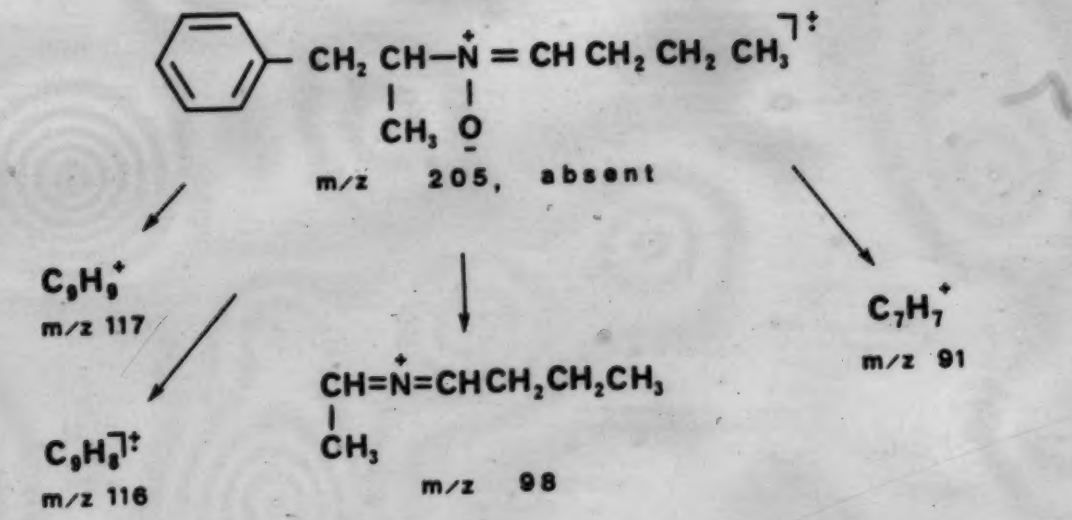


Figure 31 . The mass spectrum and fragmentation pattern of *alpha*-methyl-N-(isopropylidene)benzeneethanamine N-oxide (IOX).



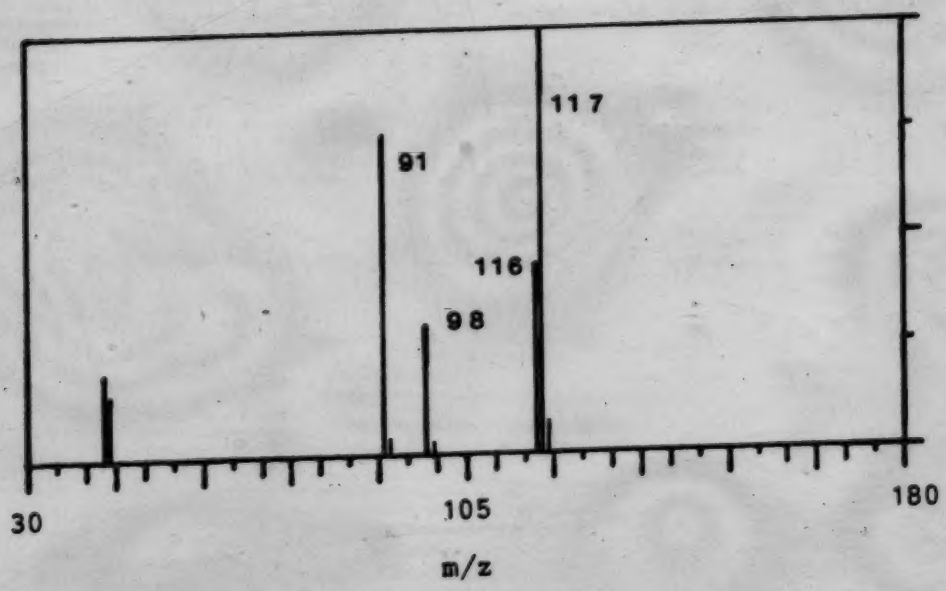


Figure 32 . The mass spectral properties of *alpha*-methyl-N-(*n*-butylidene)benzeneethanamine N-oxide (BOX).

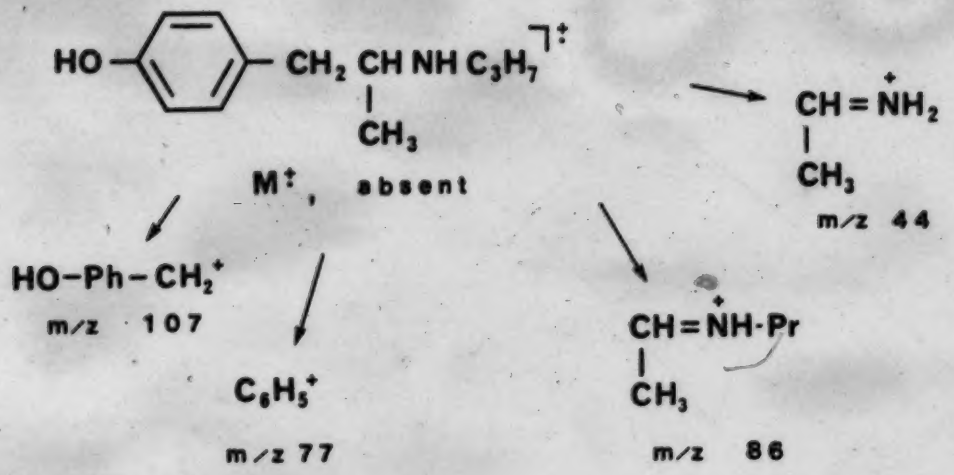
consistent with the identification of these metabolites as N-oxidation products (Beckett *et al*. 1973b; Beckett *et al*. 1974; Coutts and Beckett 1977). The presence of diagnostic ions of m/z 84 and m/z 96 in the spectra of IOX and BOX, respectively, are consistent with that expected for nitrones (Coutts and Beckett 1977). An additional N-oxidation metabolite, isolated from cultures containing NMA was identified as OXM, by comparison of its GLC and MS properties with those of authentic standard. This oxime (OXM) is a known chemical degradation product of the initial metabolite, MOX (Coutts and Kovach 1977; Coutts and Beckett 1977).

In an experiment where aliquots were examined at regular time intervals, it was found that the N-oxidation products of N-alkylamines were detectable 24 h after the addition of substrate but the N-acetylation conjugate was not detectable until a further 24 h had passed.

In a study designed to determine the relative recovery of NPA and its homologue IPA and their metabolites from *C. echinulata* incubation broths, it was evident that the relative amounts of recoverable metabolic products were different (Table 10). The major products of NPA biotransformation were AMPH, AcAMPH, *p*OH-NPA and only minor amounts of the four N-oxidation products, NOH-AMPH, OXM, NOH-NPA and NOX were detected. These results are in contrast to those previously reported in this thesis when higher quantities of N-oxidation products were detected. The metabolic profile

observed in the IPA extracts was similar to that recorded for the NPA incubation mixture although the the relative amounts of the N-oxidation products were significantly lower in the IPA extracts. There were slight differences in the GLC retention times of the corresponding NPA and IPA metabolites. The MS fragmentation patterns of the latter were virtually identical to those seen in the NPA series. The presence of m/z 86 and m/z 107 ions in the total ion trace obtained for the IPA sample is indicative of a ring hydroxylation product. This metabolite was then identified as *p*-hydroxy-N-(isopropyl)amphetamine (*p*OH-IPA, Fig. 33). The two N-oxidation products of IPA were identified as N-hydroxy-N-(isopropyl)amphetamine (IOH) and IOX. This determination was confirmed when a portion of the original extract was treated with LAH and the ensuing reaction monitored by GLC. The peak identified as IOX diminished as the reaction progressed with a concomitant increase in the IOH peak in accord with the known behaviour of nitrone reduction (Coutts and Beckett 1977).

In addition to the *C. echinulata* biotransformation products reported here and in the chapter on screening (section 4.2), a short retention time metabolite was occasionally detected in the NPA extracts. The metabolite has not been unequivocally identified but the MS fragmentation pattern is consistent with that of N-allylamphetamine (Fig. 34).



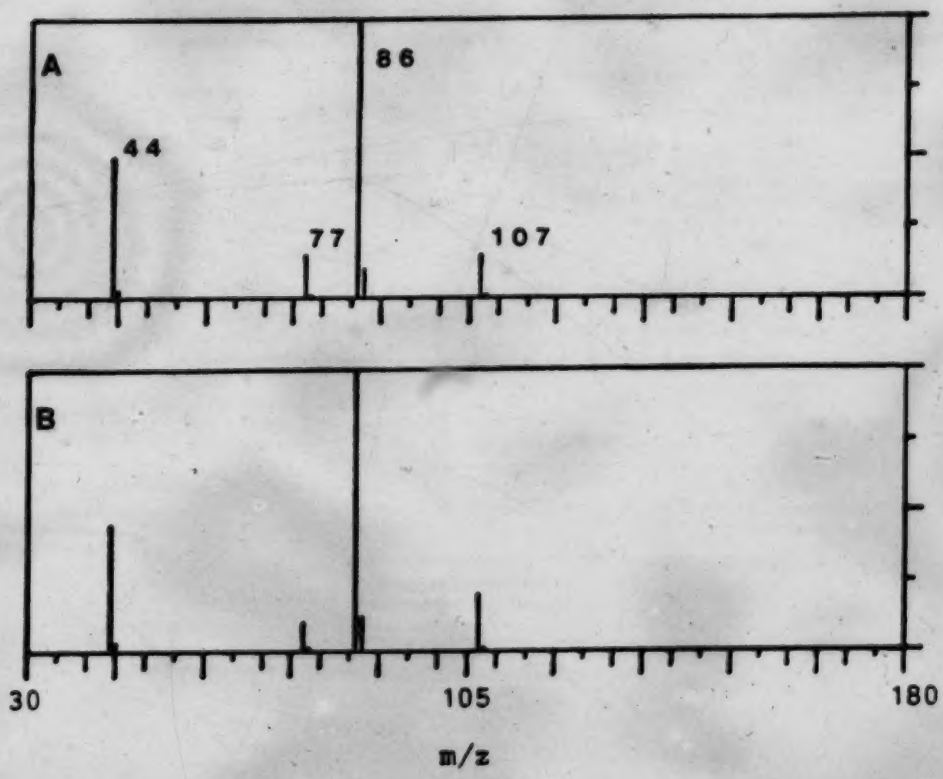
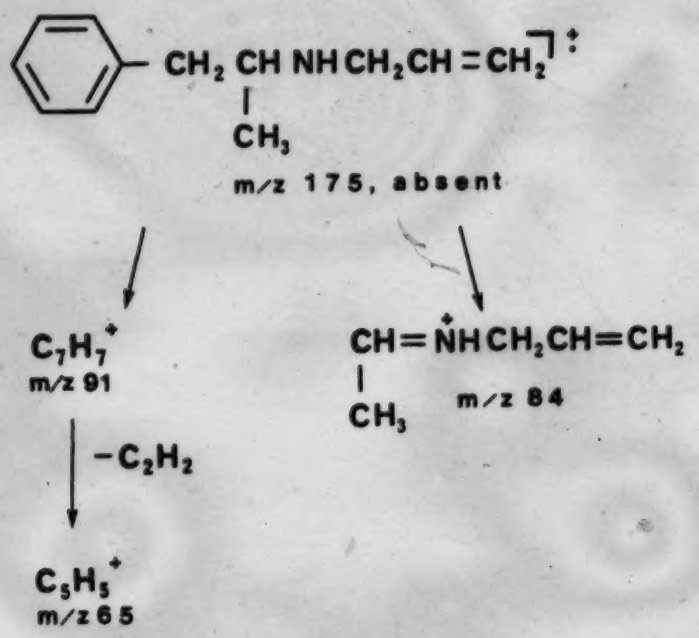


Figure 33 . The mass spectral fragmentation patterns of *p*-hydroxy-N-(isopropyl)amphetamine (A) and *p*-hydroxy-N-(*n*-propyl)amphetamine (B).



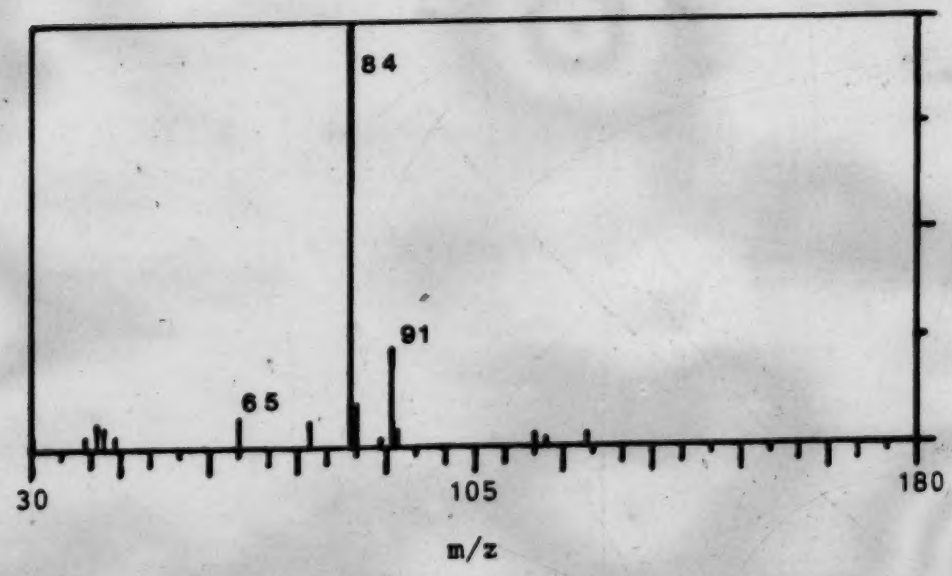


Figure 34 . The mass spectrum and fragmentation pattern of N-allylamphetamine.

Unlike *p*OH-AMPH, *p*OH-NPA and *p*OH-IPA were easily extracted from aqueous media and did not require initial aqueous acetylation for analysis. Identification of *p*OH-IPA was based on comparison of GLC-MS properties with those of the isomeric *p*OH-NPA which differed only in the GLC retention time. Fungal *p*-hydroxylation is well documented in the literature (Cerniglia and Gibson 1980; Ferris *et al*. 1973; Ferris *et al*. 1976; Smith and Rosazza 1975).

4.6.1 NPA Stereoselectivity

Studies performed with (+), (-) and (±)-NPA indicated that *M. smegmatis* has a strong metabolic preference for the (+)-isomer. NOX was the only metabolite detected in extracts of the (+) and (-)-NPA isomers; however, (+)-NOX accounted for 81% of the recoverable substrate whereas only 11% of the (-)-isomer was detected. With the racemic mixture as substrate, the nitrone accounted for 67% of the total substrate recovered. In addition, AcAMPH (6%) was also recovered.

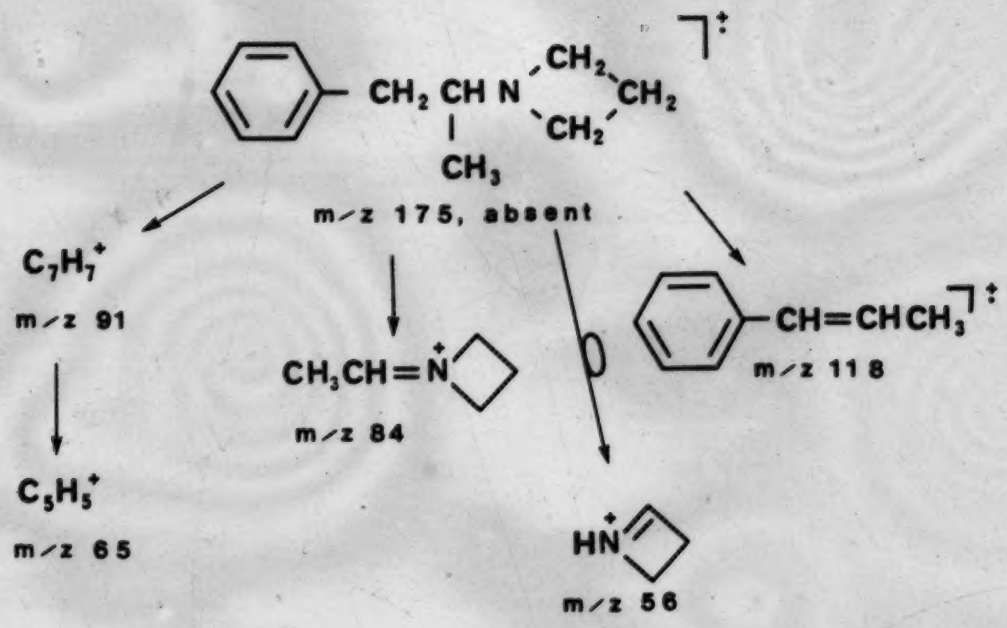
These findings are in contrast to those reported in rat liver homogenates where N-oxidation was the preferred metabolic route in the (-)-NPA isomer. C-oxidation products were the major constituents in extracts from the (+)-isomer (Coutts, Dawson and Beckett 1976).

4.6.2 NPA Homologues

In the biotransformation studies with NPA (section 4.6) it was evident that the *alpha* carbon substituent and the nature of the N-alkyl side chain influenced the course of metabolism. It was of interest to determine what effect various functional groups on the side chain would have on the overall metabolism of NPA.

Three substrates were chosen; N-(2-cyanoethyl)amphetamine (Fenproporex, FEN), N-(3-chloropropyl)amphetamine (Mefenorex, MEF) and N-(2-hydroxy-*n*-propyl)amphetamine (HPA). Stability tests demonstrated that both FEN and HPA were stable in trypticase soy broth at 27°C for 72 h but MEF decomposed. The product of decomposition was identified on the basis of its MS properties as the four membered ring derivative, N-(*alpha*-methylphenylethyl)-azetidine (MCP) (Fig. 35). The decomposition of MEF was enhanced by the alkaline conditions used in the extraction procedure (Method A, section 3.4.1) compared with neutral conditions. Recovery of MEF was higher from distilled water than from either complex medium or 0.2M phosphate buffer (pH 7.2).

In view of its instability, MEF was analyzed after 3 days and 5 days in *M. smegmatis* incubation broths. AMPH and AcAMPH were detected in extracts of day 3 samples. MEF and MCP were recovered in near equal amounts and accounted for 71% of the total recoverable substrate. The pattern shifted by day 5 when AcAMPH increased with an apparent loss of MCP. The relative amount of MEF recovered was similar to that



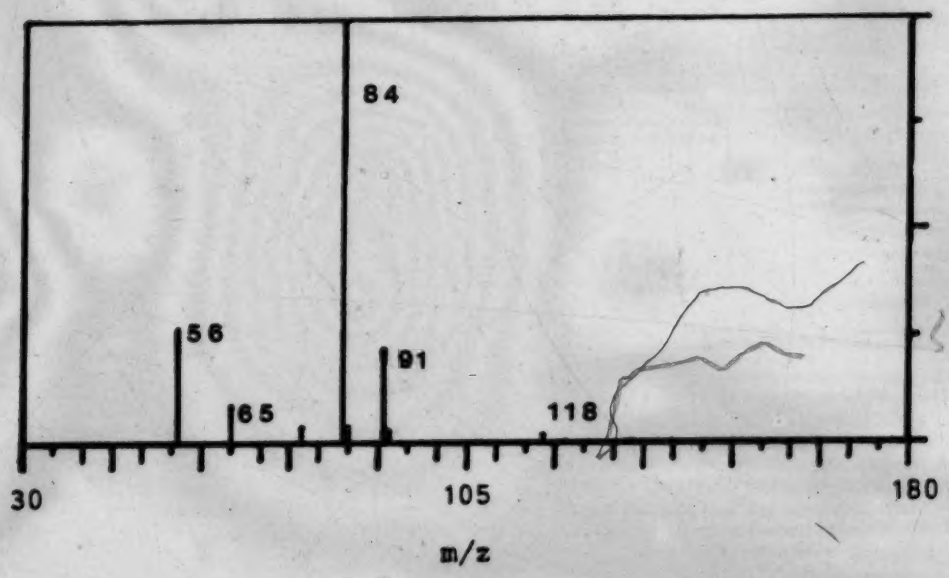


Figure 35 . The mass spectral fragmentation data of the major decomposition product of N-(2-cyanoethyl)amphetamine, N-(*alpha*-methylphenylethyl) azetidine.

seen at day 3. Occasionally, an additional peak was observed in the day 5 extract. Its MS fragmentation pattern was consistent with that expected for 2-hydroxy-N-(*alpha*-methyl phenylethyl)-azetidine (HMC, Fig. 36). However, authentic standard was unavailable to confirm the identity of this compound. HMC is an analogue of *alpha*-hydroxy -N- benzyl-cyclopropylamine, a known suicide substrate of cytochrome P-450 (Hanzlik *et al.* 1979). This agent can covalently interact with MAO after enzymatic transformation to N-benzyliminocyclopropane. The degree of inhibition with this imine is dependant on substrate concentration and does not effect NADPH cytochrome P-450 reductase nor interfere with cofactor regeneration.

Microbial systems containing a nitrilase are capable of metabolizing a nitrile to either the corresponding acid or the amide (Harper 1977)(Scheme 14).

$$R\text{-}C{\equiv}N \xrightarrow{H_2O} R\text{-}CONH_2 \xrightarrow{H_2O} RCOOH$$

Scheme 14

However, extracts (Method B, section 3.4.1) of control and *M. smegmatis* metabolic broths containing MEF were not significantly different, and neither amide nor acid was detected.

C. echinulata transformation studies with FEN, HPA and MEP were not reproducible. The results were not consistent between experiments and varying amounts of AMPH and AcAMPH were formed.

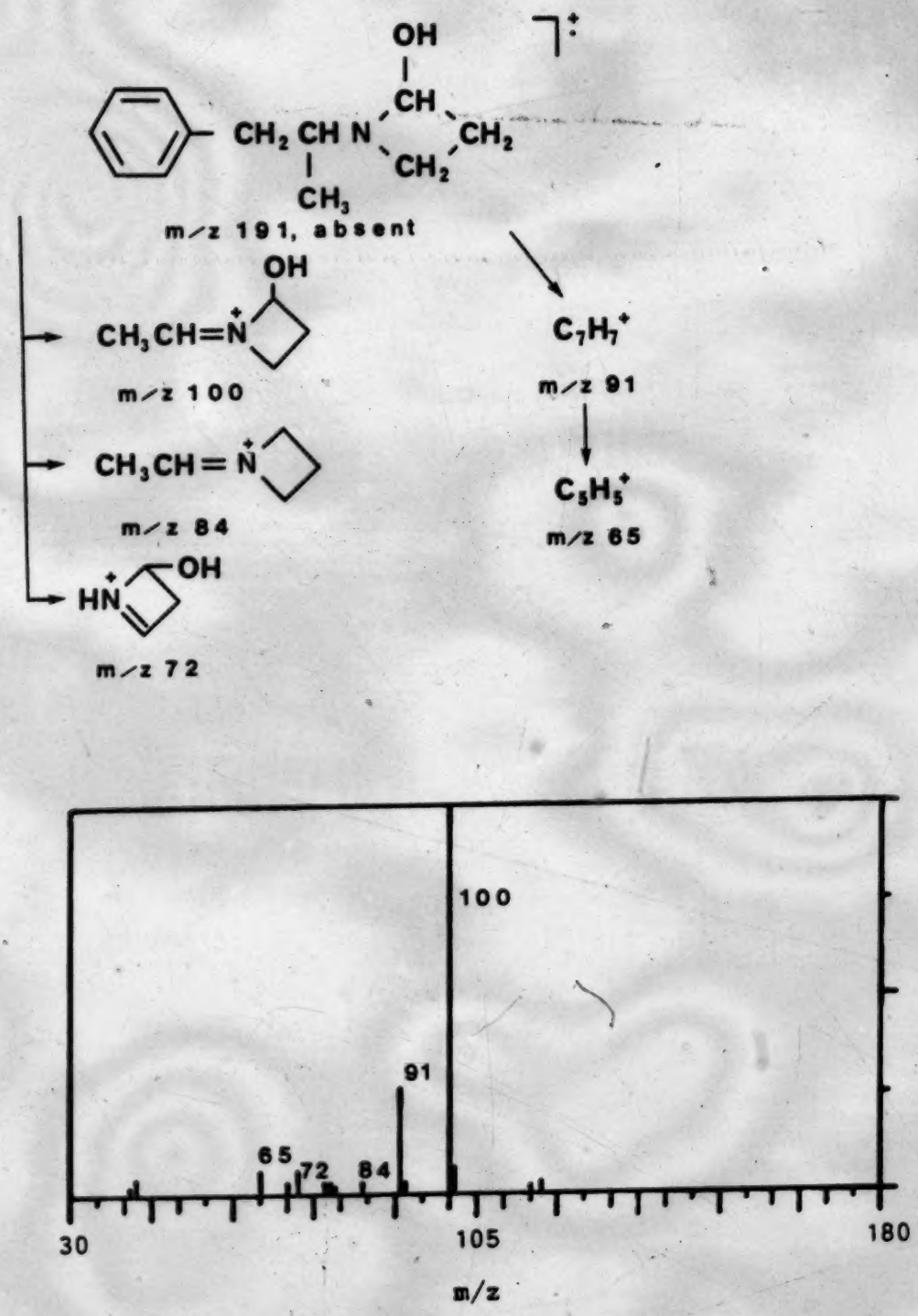


Figure 36 . Mass spectral fragmentation pattern of a product suspected to be 2-hydroxy-1-(1-methyl-2-phenyl)ethyl cyclopropylamine (HMC).

FEN was metabolized by *M. smegmatis* into two detectable products, AMPH and AcAMPH. Substrate was generally absent in extracts of day 5 samples. Recovery of FEN and its metabolites was not quantitative and GLC examination of acid extracts (Method B, section 3.4.1) were inconclusive. Benzoic acid was detected as the chloroethyl ester in both metabolic and control samples, although the metabolic sample contained slightly higher amounts of this acid.

Amphetamine was the major product of *C. echinulata* biotransformation of FEN. The extent of N-acetylation in these extracts was considerably less than in the corresponing NPA samples.

The biotransformation of FEN by *C. echinulata* and *M. smegmatis* was identical to that observed in man where FEN was metabolized faster and more extensively than NEA to AMPH (Beckett *et al.* 1972). The presence of the secondary alcohol group in N-(2-hydroxy-*n*-propyl)amphetamine hindered the metabolic biotransformation of HPA as 40% of the substrate was detected at day 5 compared with 15% recovery of NPA under identical conditions. In washed cell preparations, AMPH accounted for 3% and AcAMPH 4% of the recoverable substrate.

4.6.3 N-Oxygenated Substrates

Biotransformation of the four known N-oxidation products of NPA metabolism in mammalian systems by *M. smegmatis* was undertaken to provide additional insight into

the metabolic pathways depicted by this organism. The oxime (OXM) was slowly metabolized by *M. smegmatis* cultures to the non-nitrogenous compounds 1-phenyl-2-propanone, 1-phenyl-2-propanol and benzyl alcohol (Table 13). In a control experiment where OXM was incubated in sterile media, chemical degradation was minimal. The metabolic profile obtained was similar to that observed in rat liver suspensions where 2-nitro-1-phenylpropanone, benzylamine, and 1-phenyl-2-propanone were identified as metabolites of OXM. This result was of interest as it clarified a previously described observation (see section 4.5) that *M. smegmatis* forms only trace amounts of OXM from AMPH whereas N-oxidation is a major metabolic route with N-alkylamphetamines. The present study shows conclusively that if OXM was synthesized by this organism it would be detectable with only minor losses due to metabolism or chemical decomposition.

The metabolism of NOH-AMPH and NOH-NPA proceeded to completion. NOH-AMPH was converted mainly to AMPH, AcAMPH and the nitrone (NOX). In control experiments, both N-hydroxylated compounds underwent appreciable chemical transformation, but the major routes of chemical transformation were significantly different from the biological routes (Beckett and Achari 1977). This finding confirms that if either N-hydroxy compound was metabolically produced by *M. smegmatis* it would rapidly undergo further transformation, either biological or chemical and then

Table 13 . Biotransformation of N-oxygenated metabolites of N-(*n*-propyl)amphetamine by washed cell preparations of *M. smegmatis*.

	% yield of products'								
Substrate	A	B	C	D	E	F	G	H	I
OXM (E)	tr	8	4	20	68	-	-	-	-
control	-	1	-	6	93	-	-	-	-
NOH-AMPH (F)	54	-	-	-	9	-	-	-	37
control	4	-	-	-	82	14	-	-	-
NOX (G)	30	12	-	-	7	-	23	-	28
control	5	-	-	-	46	-	49	-	-
NOH-NPA (H)	25	-	-	-	tr	-	38	-	37
control	2	-	-	1	10	-	71	16	-

' Based on relative peak areas.

(A) amphetamine (B) benzyl alcohol (C) 1-phenyl-2-propanol (D) 1-phenyl-2-propanone (E) 1-phenyl-2-propanone oxime (F) N-hydroxyamphetamine (G) *alpha*-methyl-N-(*n*-propylidene)benzeneethanamine N-oxide (H) N-hydroxy-N-(*n*-propyl)amphetamine (I) N-acetylamphetamine.

biological, and hence would probably not be detected. It is also of interest that NOX is further metabolized by this organism to AMPH and AcAMPH.

4.6.3.1 Metabolism Modification Study

In contrast to mammalian biotransformation studies, where a great deal known concerning N-oxidation of clinically important compounds (Beckett 1971; Coutts and Beckett 1977; Gorrod 1973,1978), there is very little information available on the microbial metabolism of these compounds. The similarity between microbial and mammalian biotransformation and specificity of cytochrome P-450 reactions may make the use of micro-organisms a valuable tool to study these mechanisms further (Cooper et al 1979; Brattsten 1979; Lu 1979).

Studies on the inhibition of *M. smegmatis* metabolism were initiated in an attempt to determine what enzyme system was involved in the biotransformation of these substrates. Franklin (1974) reported that metabolism of N-substituted amphetamines was reduced by classical cytochrome P-450 inhibitors. The agents chosen for this study were carbon monoxide (CO), 2,6-dichlorophenolindophenol (DCIP), ethanol, potassium cyanide (KCN) and diethylaminoethyl-2,2-diphenyl-valerate HCl (SKF-525A) which are known to inhibit both mammalian and microbial drug metabolizing systems (Cinti 1978; Hooper 1978).

Studies on the inhibition of drug metabolism in *M. smegmatis* was hindered by the toxicity of DCIP, KCN and SKF-525A. These agents increased the tendency of this organism to clump and severely restricted growth. This reduced the number of cells in the experiment and this made it difficult to determine whether the metabolic profile observed was due to the agent or low number of cells. This problem was overcome by using washed cell preparations where the cell population did not vary during the entire experiment.

In washed cell preparations of *M. smegmatis* containing amphetamine, the relative recovery of AcAMPH was decreased 42% when the atmosphere was flushed with carbon monoxide compared to control reactions without this gas. KCN (1 mM) and SKF-525A (5 mM) reduced N-acetylation by 50 and 55%, respectively. Ethanol had no visible influence on the metabolism of AMPH.

Studies on the inhibition of NPA metabolism revealed that the addition of KCN or ethanol to the washed cell preparations of *M. smegmatis* reduced nitrone (NOX) formation by 60 and 78%, respectively (Table 14). The addition of DCIP resulted in complete inhibition of N-oxidation. SKF-525A decreased the relative amount of NOX formed by 78%. When the washed cell preparations were incubated in an atmosphere containing 20-50% carbon monoxide, NOX formation was reduced by 80% when compared to control results.

Table 14 . Effect of metabolic inhibitors on the biotransformation of N-(*n*-propyl)amphetamine by *M. smegmatis*.

Substrate	Recovered substrate	% yield of isolated products[1] AMPH	NOX	AcAMPH
Control[2]	75	8	14	3
Ethanol, 4%	95	tr	3	2
KCN, 1 mM	95	-	5	tr
DCIP, 1 mM	100	tr	tr	tr
Control[3]	86	tr	14	-
CO	97	-	3	-
SKF-525A, 5 mM	96	tr	4	-

[1] Based on relative peak area. [2] NPA was added at a concentration of 7 mg/flask. [3] NPA (2 mg/flask) was added to washed cells in phosphate buffer. (tr) trace. (KCN) potassium cyanide (DCIP) 2,6-dichlorophenolindophenol (CO) carbon monoxide.

In washed cell preparations with NOH-NPA as substrate, the relative amounts of recoverable metabolites (AcAMPH 61% and NOX 39%), were reversed by the addition of ethanol.

Ethanol is a powerful hydroxyl radical scavenger and can compete with substrates for hydroxyl radicals (Cohen and Cederbaum 1980). Inhibition of metabolite formation in incubation broths containing ethanol would indicate that substrate oxidation was in part due to peroxidase activation (Coon 1981). SKF-525A inhibits only hemoproteins and simultaneous addition of SKF-525A and drug results in competitive competion for the same binding sites. The extent to which SKF-525A inhibits biotransformation is dependant on the metabolism of SKF-525A (Schenkman *et al.* 1972). Addition of the substrate prior to SKF-525A results in higher levels of transformation. Inhibition by this agent would indicate involvement of a hemoprotein, such as cytochrome P-450 (Lu and West 1980; Schenkman *et al.* 1972). DCIP can interfere with cytochrome mediated transformation by disrupting the flow of electrons (Hooper 1978). Inhibition of biotransformation by KCN and carbon monoxide provides strong evidence that N-oxidation is mediated by a cytochrome P-450 like system and not a flavoprotein oxidase (Coon *et al.* 1977; Ziegler and Mitchell 1972).

The inhibitory effects of these agents collectively establishes that a cytochrome P-450 like drug metabolism system is involved in the C- and N-oxidations observed with these substrates. There are two mechanisms which may account

for the formation of NOX (Fig. 37). The studies of Beckett and Belanger (1974) with liver homogenates suggested that a peroxidase was involved in the formation of a N-hydroperoxide intermediate from a secondary amine. Subsequent dehydration of the N-hydroperoxide gave rise to a nitrone. The alternate mechanism required that a mixed function monooxygenase forms a hydroxylamine intermediate which underwent dehydrogenation to a nitrone. If this mechanism prevailed in *M. smegmatis* it should be possible to detect the hydroxylamine intermediate. In the present study, no hydroxylamine intermediates were isolated. This does not eliminate the feasibility of either mechanism; the hydroxylamine intermediate may be bound to the enzyme and rapidly metabolized to the nitrone or as Coon (1980) has shown, cytochrome P-450 may function as either a peroxidase or mixed function monooxygenase depending on environmental factors. Hence, in this study either mechanism may be involved in the N-oxidation of these substrates, although the data does not preclude the possibility of additional pathways.

The classical enhancers of cytochrome P-450 activity, phenobarbital (PB) and 3-methylcholanthrene (MC) were added to growing cultures of *M. smegmatis* (stage I) to determine whether the relative amounts of N-oxidation products could be altered. PB (100 mg/L) and MC (25 mg/L) did not significantly alter the metabolic profile of *M. smegmatis* metabolism of either AMPH or NPA at the usual drug dosage.

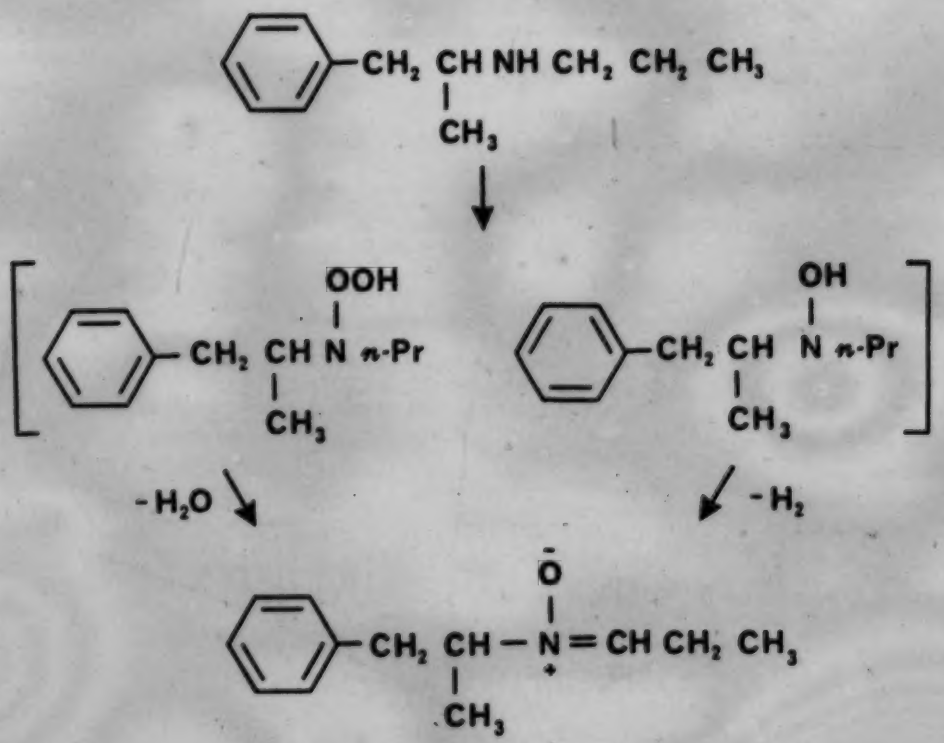


Figure 37 . Possible routes of N-(*n*-propyl)amphetamine N-oxidation to *alpha*-methyl-N-(*n*-propylidene) benzeneethanamine N-oxide by *M. smegmatis*.

If the substrate concentration was increased twenty fold the level of N-acetylation increased by 2-3 fold. These findings are in agreement with those of Callen (1978) that in micro-organisms, cytochrome P-450 induction by classical mammalian agents may not be significant. It is more common for induction and inhibition of microbial enzymes to occur in the presence of a particular carbon or nitrogen source (Aharonowitz 1980; Aharonowitz and Demain 1980; Callen 1978; Rose 1979).

4.6.4 HPLC of N-hydroxyamphetamine Metabolites

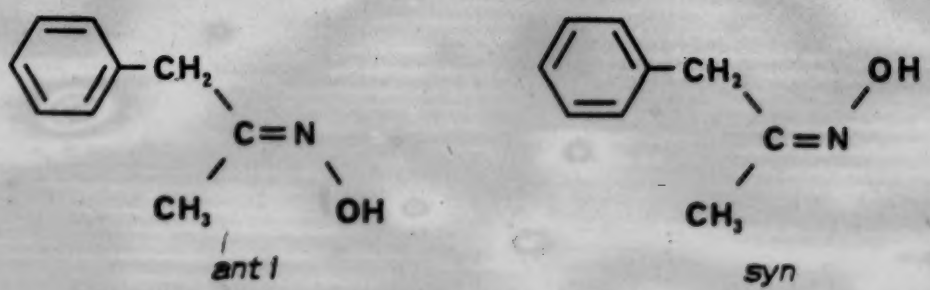
Extracts of *M. smegmatis* incubation broths containing NOH-AMPH were analyzed by GLC. In addition to NOH-AMPH, OXM, AcAMPH and trace amounts of 1-phenyl-2-propanone were detected. The known chemical decomposition pattern (Scheme 15 , Coutts and Beckett 1977) of this substrate also give a similar pattern of products except for AcAMPH,

$$Ph\text{-}CH_2CH(CH_3)NHOH \longrightarrow Ph\text{-}CH_2C(CH_3)=NOH \longrightarrow Ph\text{-}CH_2COCH_3$$

Scheme 15

but, the relative amounts of each product were different from those observed in the metabolic extracts (Method A, section 3.4.1)(Table 11). HPLC analysis of control extracts containing NOH-AMPH revealed the presence of three products of NOH-AMPH decomposition. These were 1-phenyl-2-propanone and the two isomers of OXM. The OXM anti isomer was the major chemical decomposition product of this substrate.

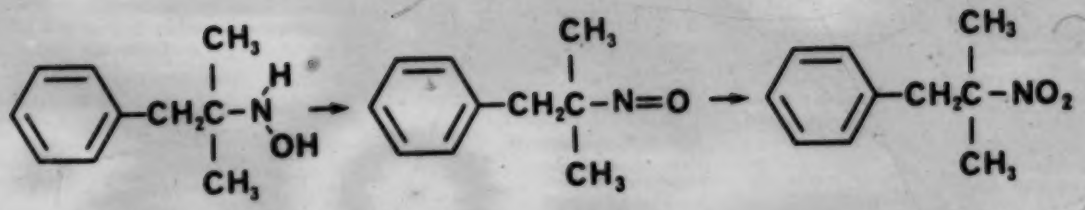
It was of interest that HPLC analysis of extracts from metabolism studies with this organism revealed that the OXM



syn isomer was the major product of NOH-AMPH biotransformation with only minor amounts of the anti isomer being formed. HPLC analysis can be utilized to differentiate between the chemical and biological transformation routes of NOH-AMPH.

4.7 Phentermines

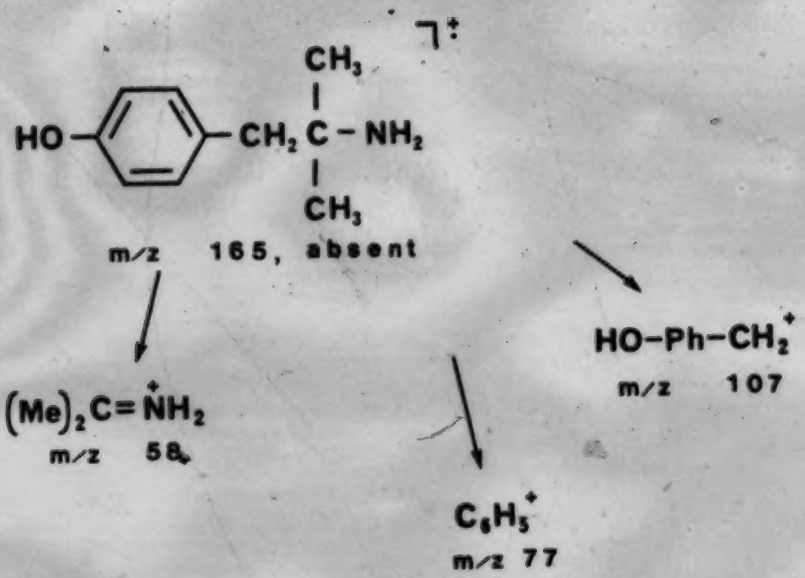
Phentermine (PHT) is a anorexic agent and analogue of AMPH. This substrate was studied to determine whether the presence of an additional *alpha*-methyl group would influence microbial biotransformation. This was of special interest as PHT is metabolized in man and laboratory animals to N-hydroxyphentermine and *alpha,alpha* dimethyl-*alpha*-nitroso-β-phenylethane (Beckett and Belanger 1974, 1978; Cho *et al*. 1974; Sum and Cho 1977, 1979). It was determined that in the alkaline conditions required for extraction, the hydroxylamine was converted to the nitroso product which was subsequently oxidized chemically to the nitro derivative, *alpha,alpha*-dimethyl-*alpha*-nitro-β-phenylethane (Scheme 16.) (Beckett and Belanger 1974). The ring hydroxylation product, *p*-hydroxyphentermine (*p*OH-PHT), a minor metabolite of PHT in man (Cho 1974) and is the only other known metabolite of this substrate.



Scheme 16

Metabolism of PHT, N-(*n*-propyl)phentermine (NPT), and N-(isopropyl)phentermine (IPT) in *C. echinulata* cultures produced the products identified in Table 10. Substrate was recovered in all instances. The only metabolite obtained from PHT metabolism had GLC characteristics compatible with a ring hydroxylated compound. MS analysis of this metabolite revealed the presence of diagnostic ions consistent with those expected for *p*-hydroxyphentermine (*p*OH-PHT, Fig. 38). A portion of the original extract containing this metabolite was allowed to react with acetic anhydride. The sample was analyzed and the peak tentatively identified as *p*OH-PHT was absent from the GLC trace and a new peak was subsequently detected (Fig. 39). Selective cleavage of the sample with 7N ammonium hydroxide resulted in O-deacetylation of the ring acetyl group. The GLC/MS properties of authentic *p*OH-PHT and its acetylated derivatives were identical to those of the metabolite and its acetate.

Significant quantities of *p*OH-PHT were isolated from cultures containing NPT and IPT. In addition, a second metabolite was detected in each extract. The MS



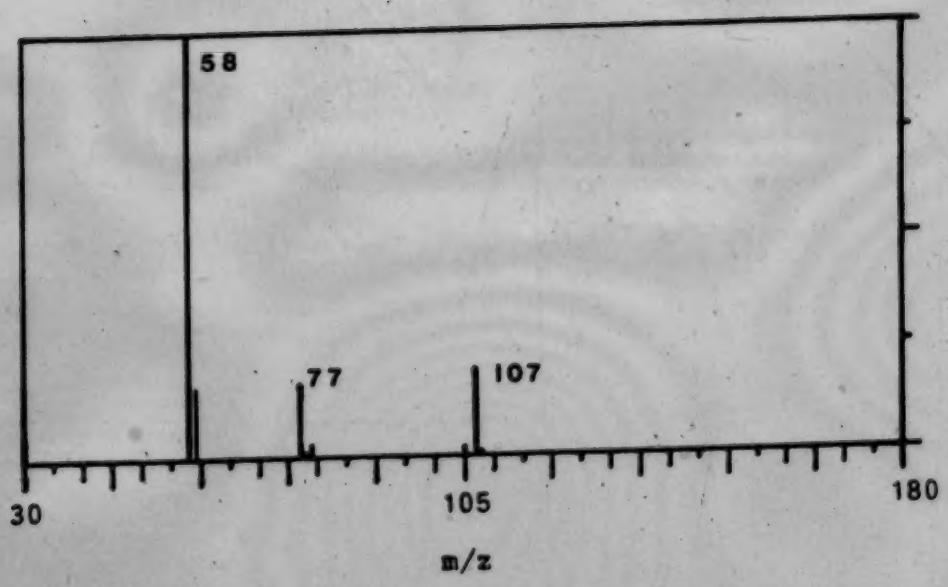


Figure 38 . The mass spectral properites of *p*-hydroxyphentermine.

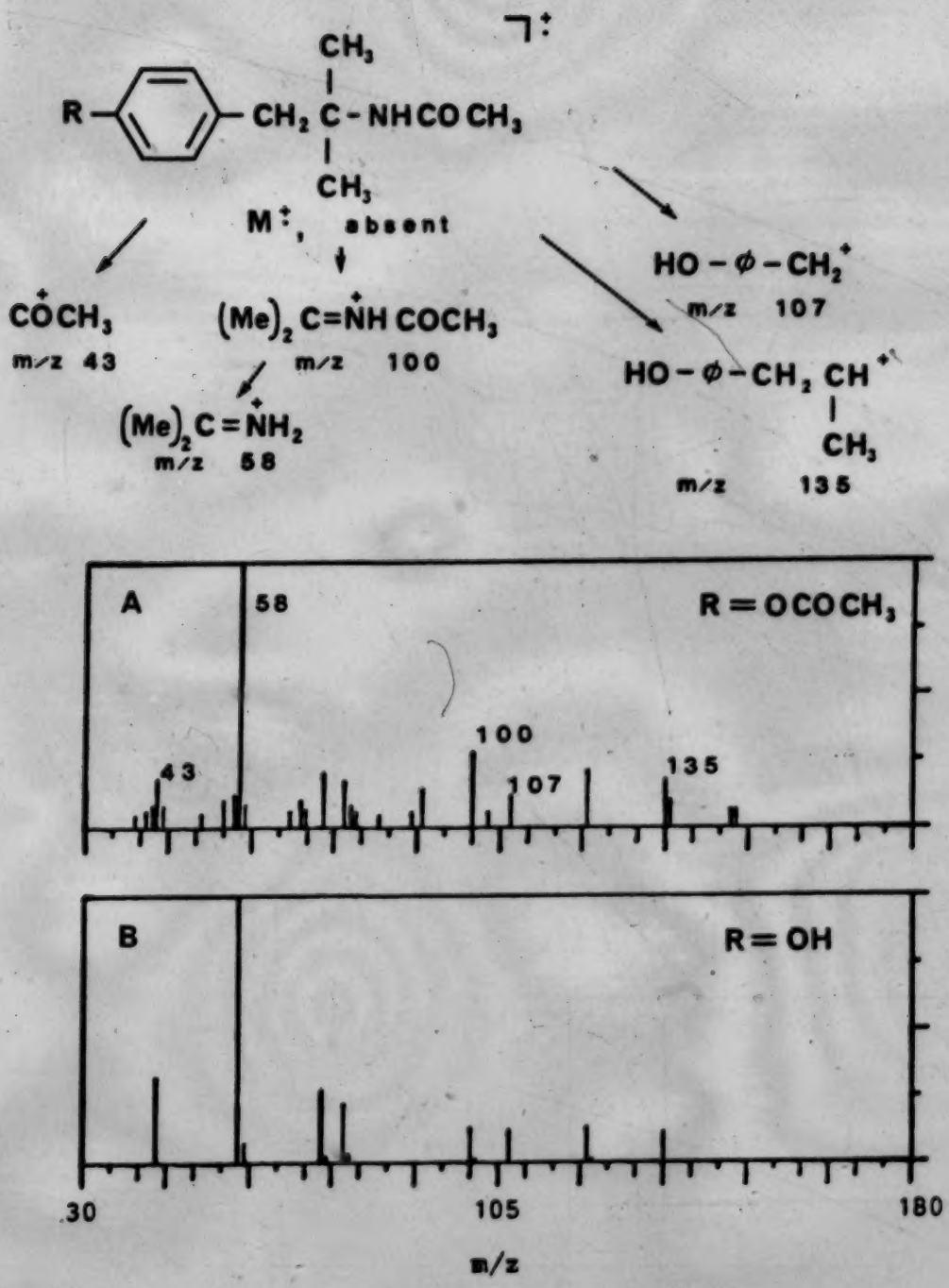


Figure 39 . The mass spectra of the *p*-hydroxyphentermine products of acetylation and O-acetyl cleavage. (A) N,O-diacetyl-*p*-hydroxyphentermine (B) N-acetyl-*p*-hydroxyphentermine.

fragmentation pattern of these metabolites were identical, although their GLC retention times were considerably different. These products and their acetate derivatives were identified by interpretation of their mass spectra as *p*-hydroxy-N-(*n*-propyl)phentermine (*p*OH-NPT) and *p*-hydroxy-N-(isopropyl)phentermine (*p*OH-IPT) (Fig. 40).

Acetic anhydride treatment of the incubation broths before extraction did not result in an increased recovery of the ring hydroxylated products of these phentermines. This contrasted earlier findings with *p*-tyramine and *p*-hydroxyamphetamine which required acetylation prior to extraction to ensure complete recovery of these phenolic amines.

Recovery of PHT from *M. smegmatis* incubation broths was virtually quantitative and no metabolites were observed (Table 11). Examination of IPT incubation extracts revealed that this substrate also was not metabolized by this organism. In extracts of broths containing N-(ethyl)phentermine (NET), NPT and N-(*n*-butyl)phentermine (NBT) two metabolites were detected. The N-dealkylation product, PHT, was a major metabolite in each extract. The MS fragmentation patterns of the remaining metabolite in the extract of NET (Fig. 41), NPT (Fig. 42), and NBT (Fig. 43) was consistent with that expected for a nitrone (Coutts and Beckett 1977). A portion of the original extract of these substrates was treated with LAH. The ensuing reaction was monitored by GLC. The peak which was tentatively identified as the nitrone decreased in peak area with the concomitant

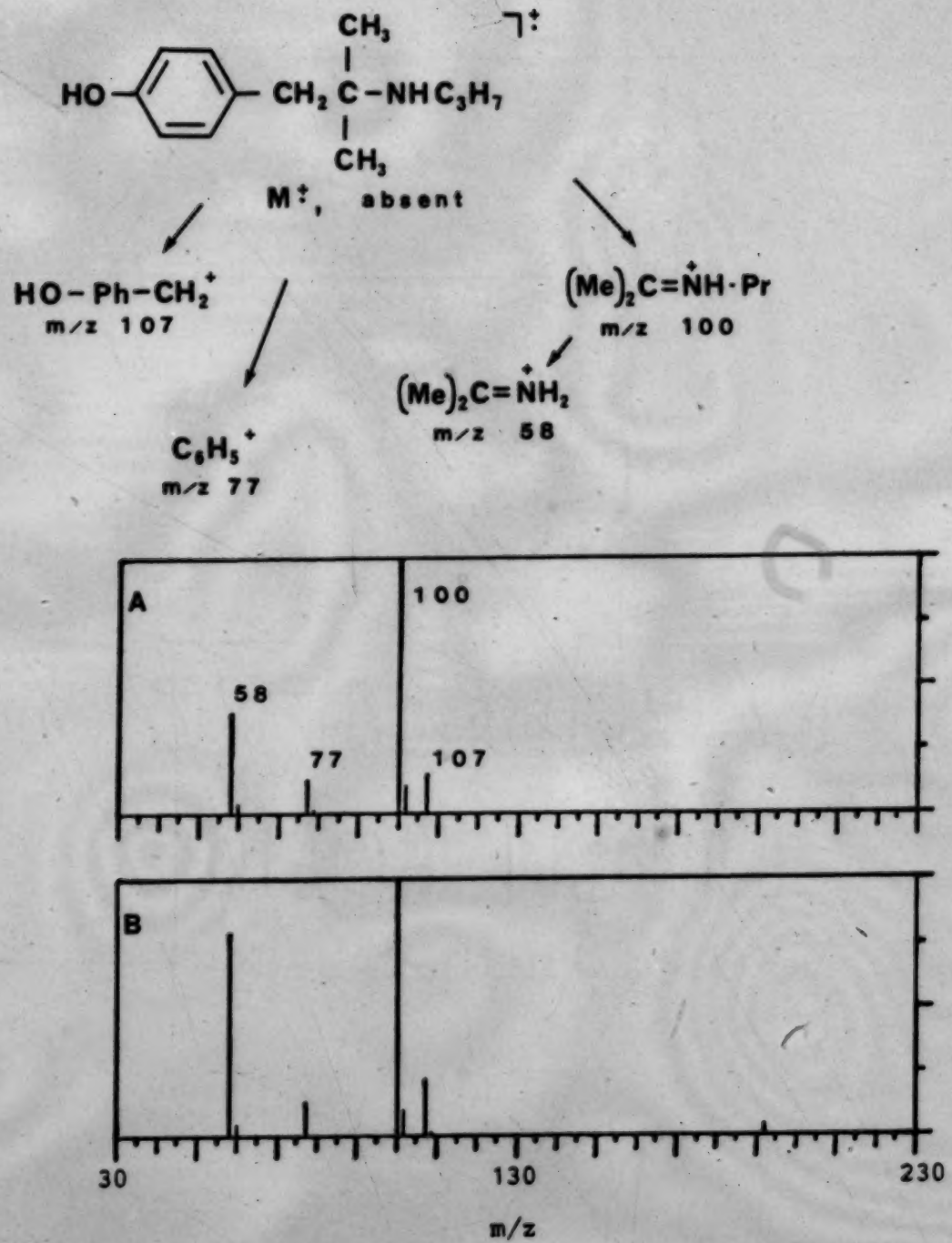


Figure 40 . The mass spectra of *p*-hydroxy-N-(*n*-propyl)phentermine (A) and *p*-hydroxy-N-(isopropyl)phentermine (B).

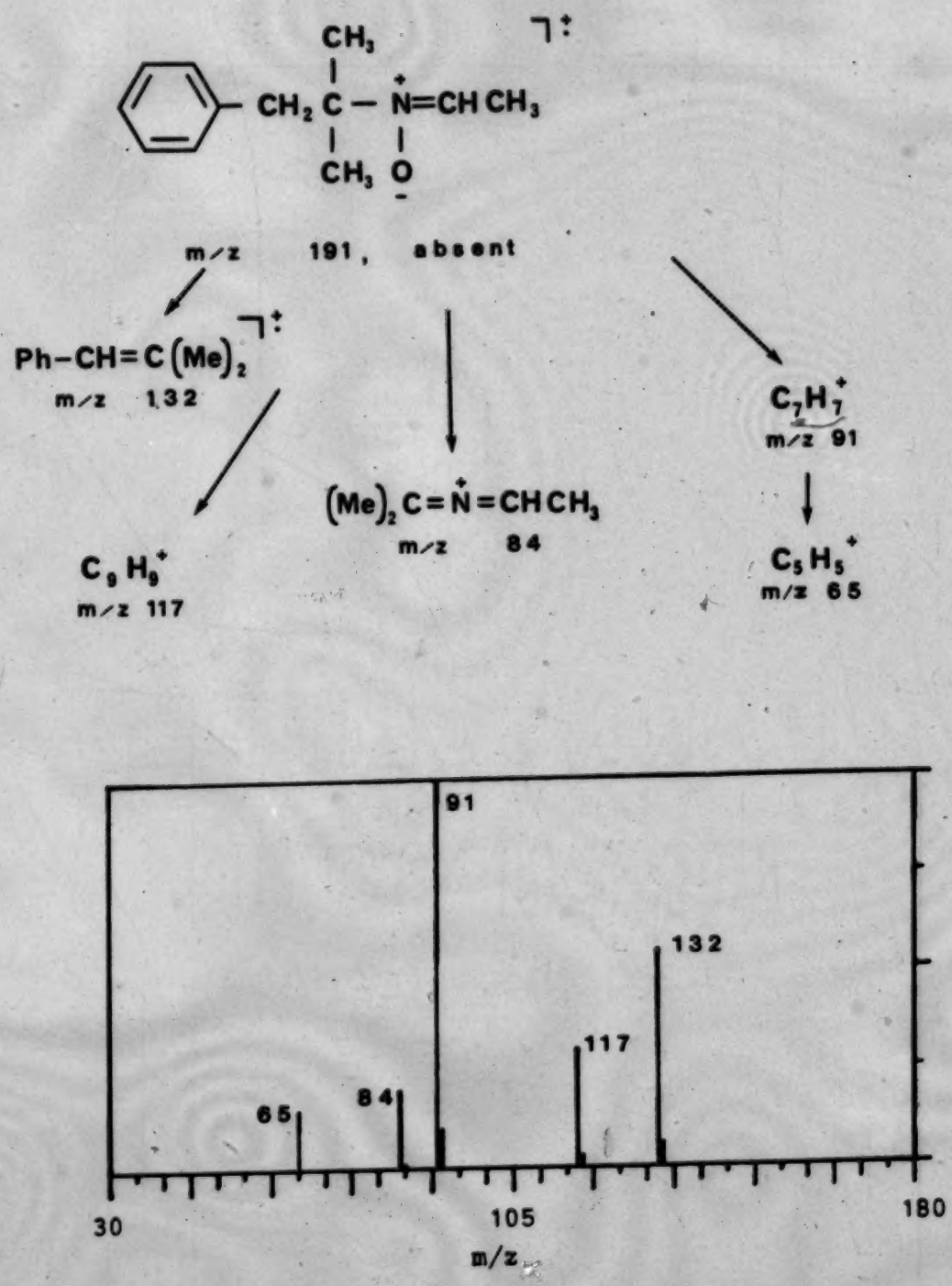


Figure 41 . *alpha,alpha*-dimethyl-N-(ethylidene)benzene-ethanamine N-oxide mass spectral properties.

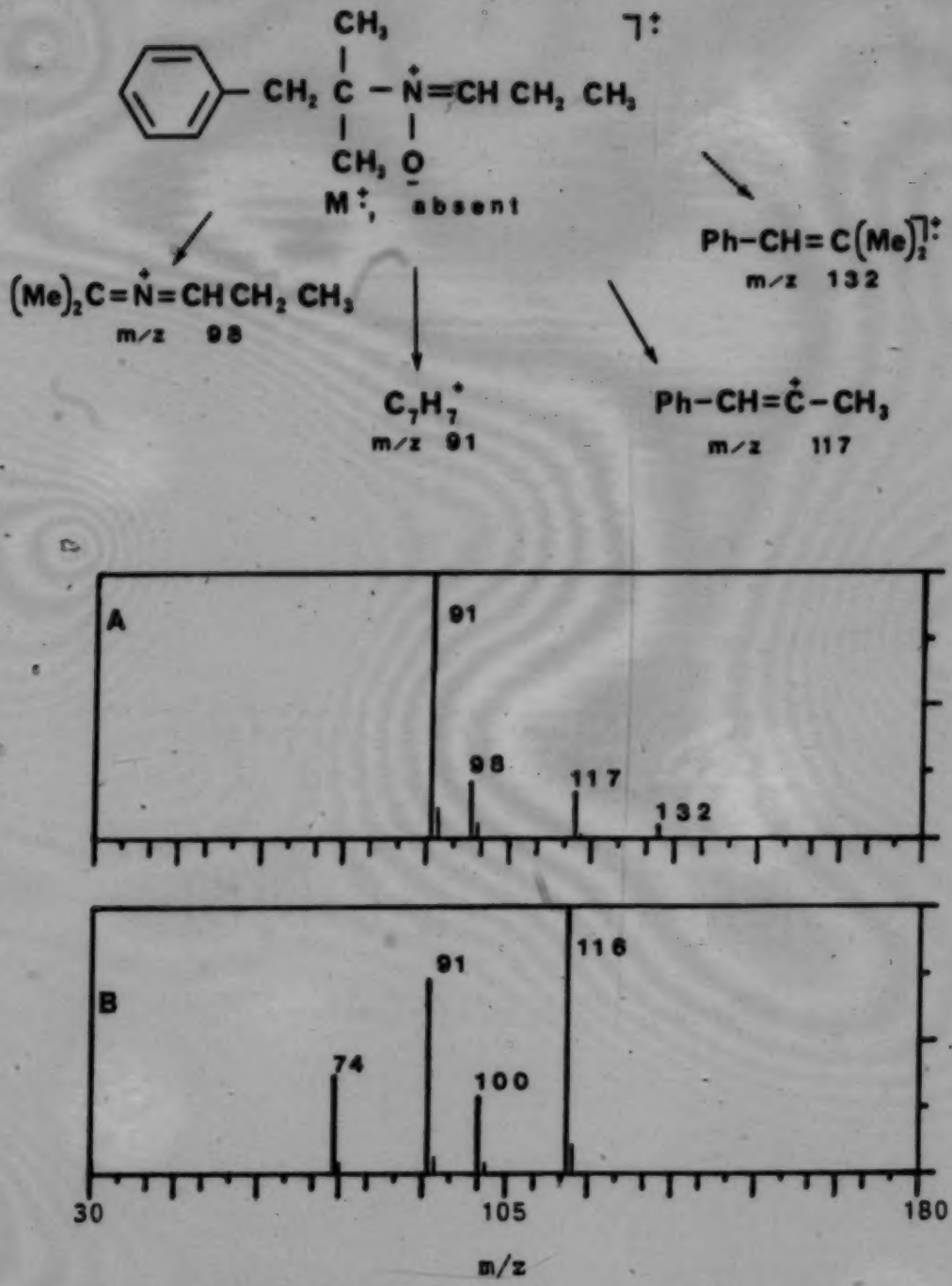


Figure 42 . Mass spectral properties of *alpha,alpha*-dimethyl-N-(*n*-propylidene)benzeneethanamine N-oxide (NTOX) (A) and its LAH reduction product (B).

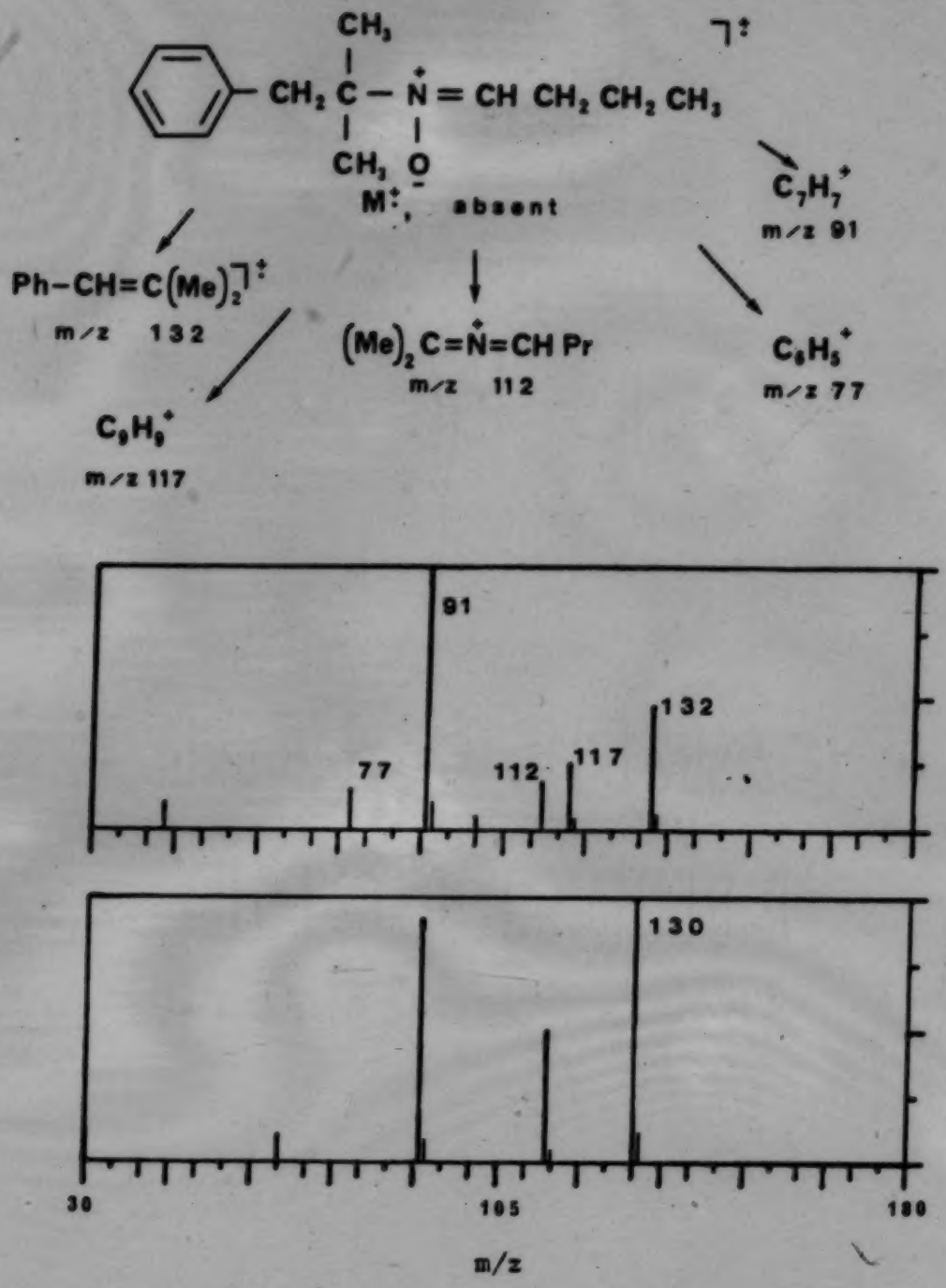


Figure 43 . Mass spectral properties of *alpha,alpha*-dimethyl-N-(*n*-butylidene)benzeneethanamine N-oxide (BTOX) (A) and its LAH reduction product (B).

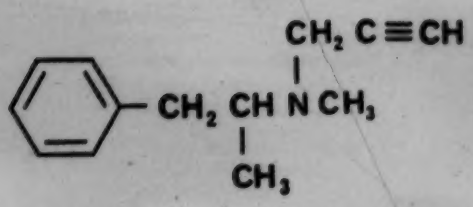
formation of a new compound. The GLC/MS properties of the new peaks were consistent with those expected for the N-hydroxylated products (Coutts and Beckett 1977).

The identification of each new metabolite was readily confirmed to be the nitrone of NET, NPT and NBT (*alpha,alpha*-dimethyl-N-(ethylidene)benzeneethanamine N-oxide (ETOX), *alpha,alpha*-dimethyl-N-(*n*-propylidene)-benzeneethanamine N-oxide (NTOX) and *alpha,alpha*-dimethyl-N-(*n*-butylidene)benzeneethanamine N-oxide (BTOX), respectively).

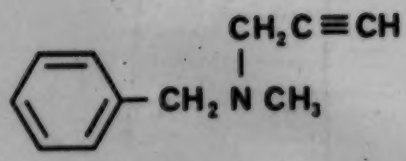
It was of interest that the N-acetylated derivative of PHT was not detected in any extracts from either *C. echinulata* or *M. smegmatis* metabolism of PHT and its N-alkyl homologues.

From these studies it is evident that there are differences in the N-oxygenases present in *C. echinulata* and *M. smegmatis*. The *M. smegmatis* system was able to effect N-oxidation of NET, NPT and NBT but not PHT or IPT. In contrast, *C. echinulata* was unable to mediate N-oxidation of any of these substrates. This finding emphasizes that there are alternate metabolic routes available to these micro-organisms and that the absence or presence of a particular metabolite may reflect the predominance of one route over another.

4.8 Aliphatic Acetylenic Amines



(-)-Deprenyl



Pargyline

Acetylenic amines are a class of chemical compounds for which biotransformation mechanisms have not been adequately studied despite the fact that a number of them are pharmacologically active monoamine oxidase inhibitors (MAOI) and are used clinically. (-)-Deprenyl is a MAOI with selective action against the B form of the enzyme, monoamine oxidase (MAO:E.C.1.4.3.4). Its administration prevents degradation of dopamine and other brain amines (Reynolds *et al*. 1978). Pargyline is a MAOI, anti-hypertensive agent and inhibitor of aldehyde dehydrogenase (AlDH) (Shirota *et al*. 1979, 1980). Pargyline at high but not low dose can significantly reduce the rat hepatic microsomal ethylmorphine N-demethylase activity and cytochrome P-450 content (Valerino *et al*. 1978). While the microbial metabolism of primary and secondary arylalkylamines has been studied, very little is known concerning the biotransformation of tertiary amines containing an N-propynyl group (Reynolds *et al*. 1978).

4.8.1 Microbial metabolism

Incubation broths containing *C. echinulata* and pargyline (PAR) were extracted with a mixture of diethyl ether-methylene chloride to obtain neutral and alkaline metabolites and recoverable substrate (Method A, section 3.4.1). The extract was analyzed by GLC and found to contain substrate, identified as A, and five metabolites, identified as B-F (Fig. 44). Metabolites B, D and F were readily revealed, by their mass spectral behavior and by direct comparison of their GLC-MS properties (Table 15) with authentic samples, to be benzylamine (BEN), N-propargyl-benzylamine (PBA) and N-acetylbenzylamine (AcBEN) (Fig. 45). Metabolite C was identified on the basis of reported mass spectral properties (Pirisino *et al.* 1979) as N-methyl-benzylamine (MBA). A plausible structure for metabolite E was deduced by interpretation of its mass spectrum to be the N-oxidation product of PBA, N-hydroxy-N-propargylbenzylamine (NOH-PBA, Fig. 45 and 46). A portion of the original extract was reduced to dryness and derivatized with BSA. The silylated products were analyzed by GLC/MS (column C, 100°C, section 3.4.5) in the selected ion monitoring mode. The product corresponding to metabolite E was detected and studied further by GLC/MS analysis. The presence of the molecular ion (m/z 233) and fragmentation ions m/z 142 and m/z 218 (Fig. 47)in the silyl derivative of E were consistent with the identification of this metabolite as the N-hydroxylation product, NOH-PBA. Confirmation of the

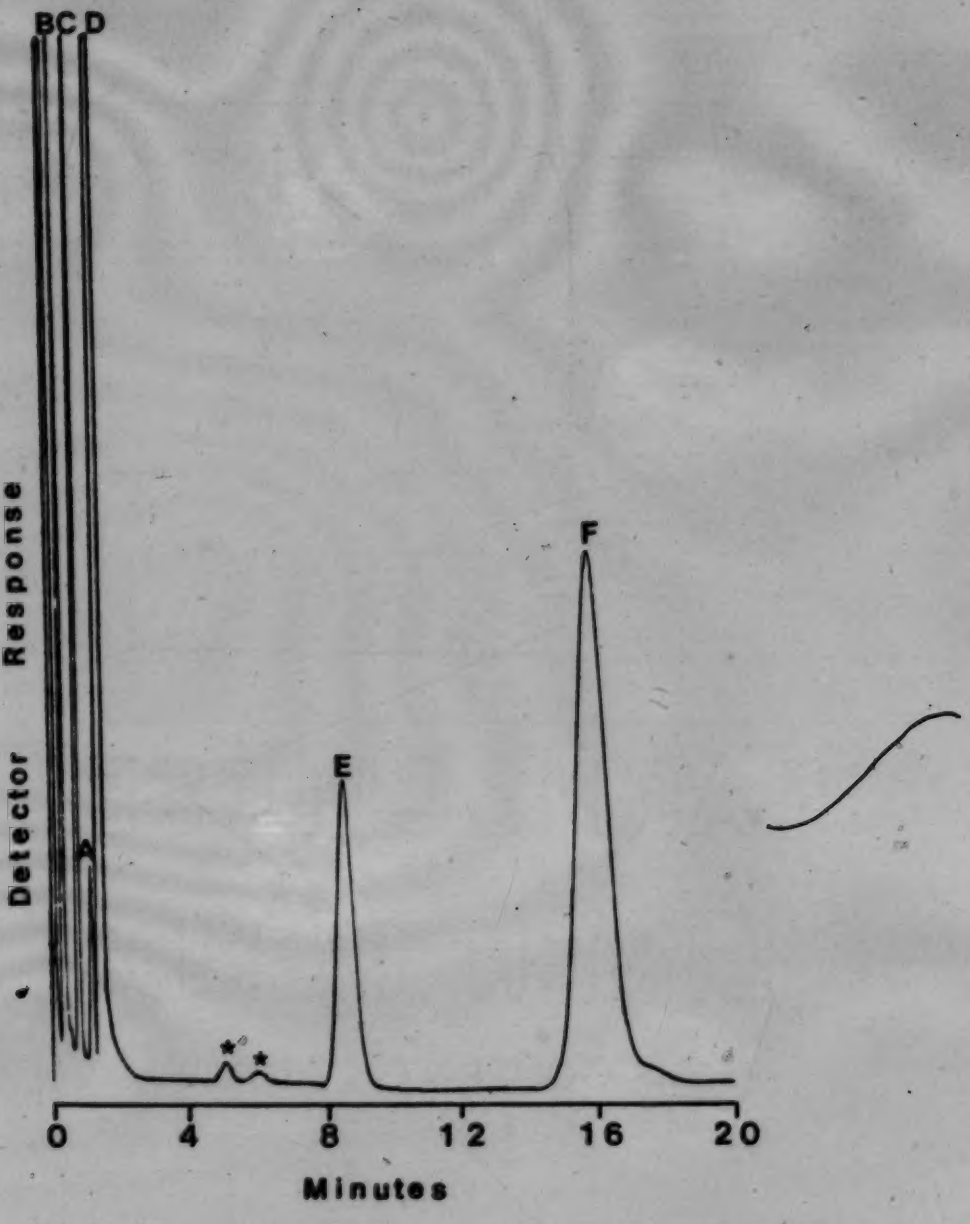


Figure 44 . GLC trace of a pargyline metabolic extract. (A) pargyline, (B) benzylamine, (C) N-methylbenzylamine, (D) N-propargylbenzylamine, (E) N-hydroxy-N-propargylbenzylamine, and (F) N-acetylbenzylamine. * unidentified metabolites. System B, 145°C.

Table 15 . Gas-liquid chromatography retention times (Rt) and mass spectral fragmentation data of deprenyl and pargyline and their metabolites.

Compound	Rt(min) A'	B	MS Data, m/z (% Relative abundance)
PAR	1.45	1.35	159(70), 158(57), 91(100), 82(59)
BEN	.70	.50	107(60), 106(100), 91(20), 79(35)
MBA	.80	.90	121(60), 120(100), 91(51), 77(10)
PBA	1.47	1.50	145(24), 144(100), 104(20), 92(26)
NOH-PBA	-	8.60	161(19), 145(5), 144(6), 91(100)
AcBEN	2.90	15.60	149(100), 107(18), 106(97), 91(24)
DEP	3.20	1.90	96(90), 91(100), 77(5), 65(5), 56(11)
AMPH	1.04	.60	135(2), 91(56), 44(100)
NMA	1.27	.84	148(3), 91(26), 58(100)
OXIME	-	5.50	149(77), 117(31), 116(51), 91(100)
AcAMPH	4.60	12.00	118(65), 91(35), 86(100), 44(18)

'GLC columns are described in Materials and Methods.
Oven temperatures: column A, 155°C; column B, 145°C.

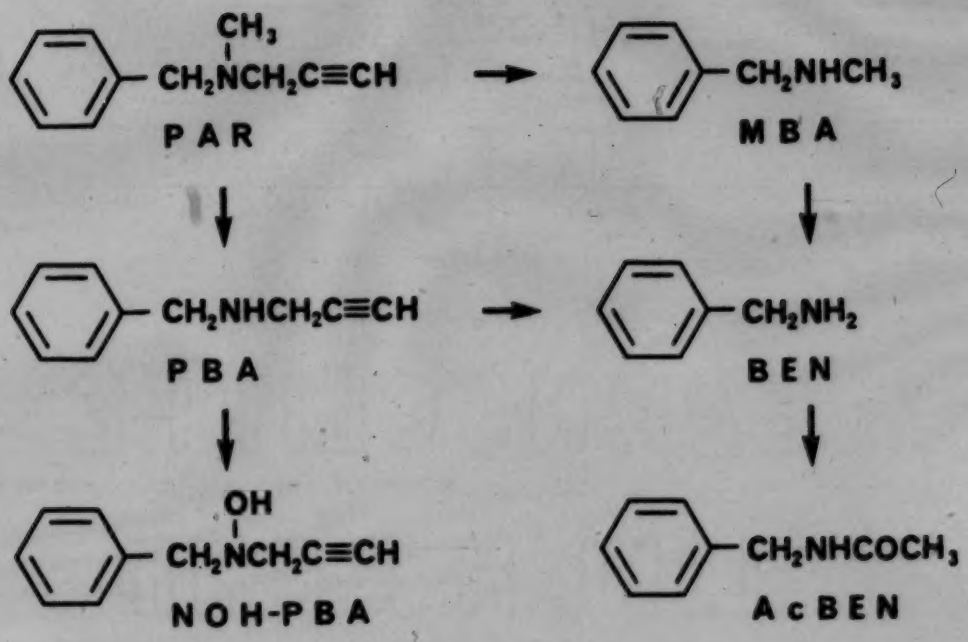
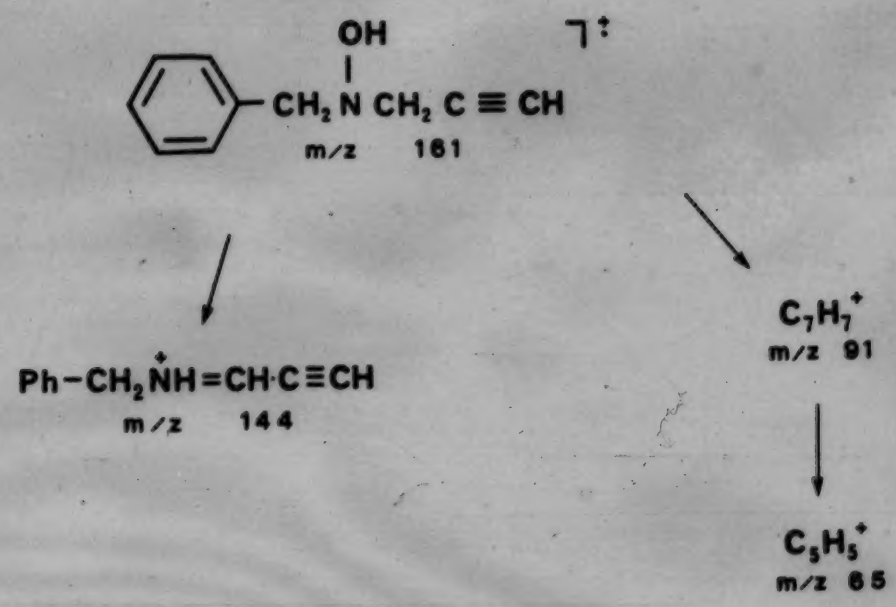


Figure 45 . Routes of pargyline metabolism in *C. echinulata*.



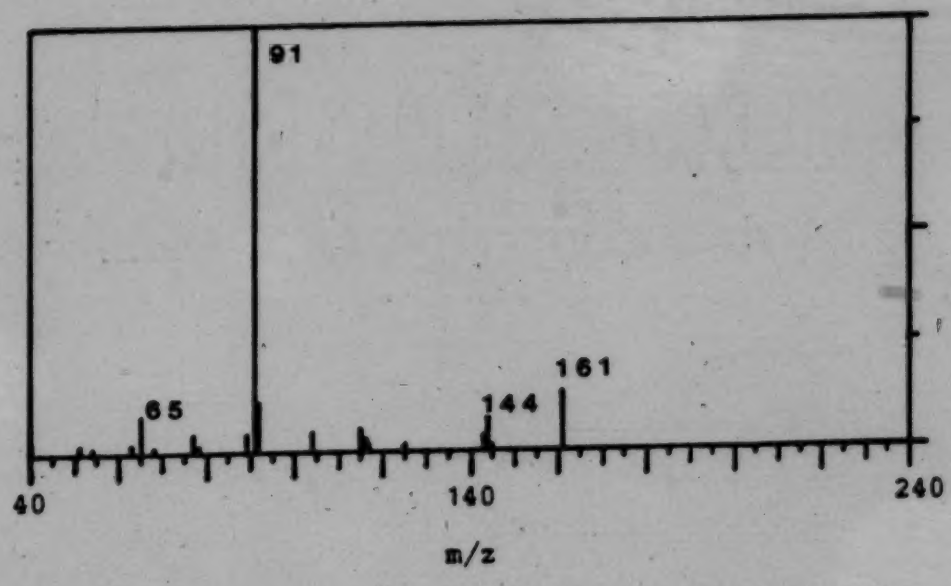
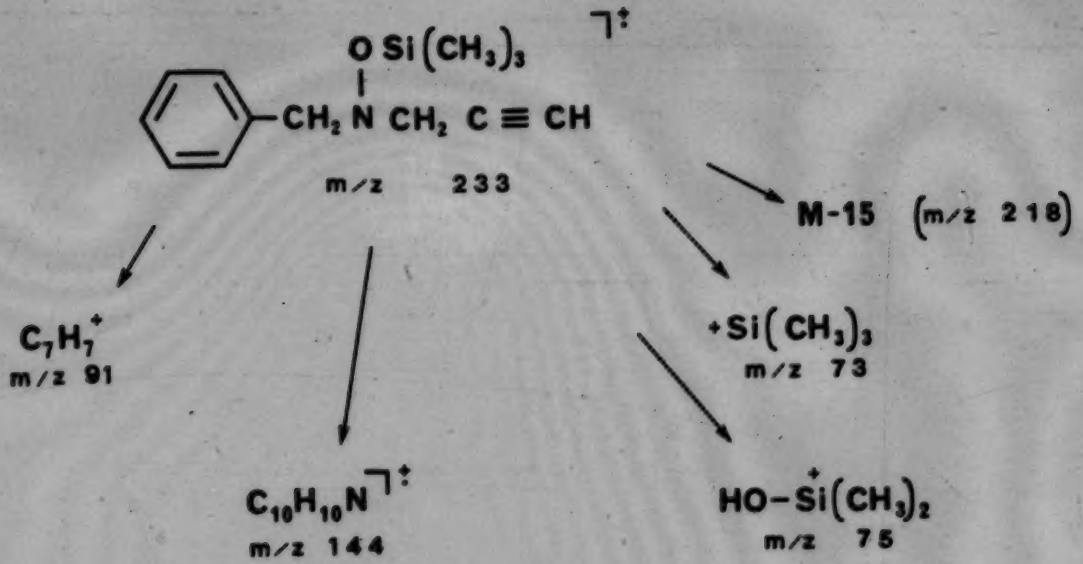


Figure 46 . The mass spectral properties of the N-oxidation product of N-propargylbenzylamine, N-hydroxy-N-propargyl-benzylamine.



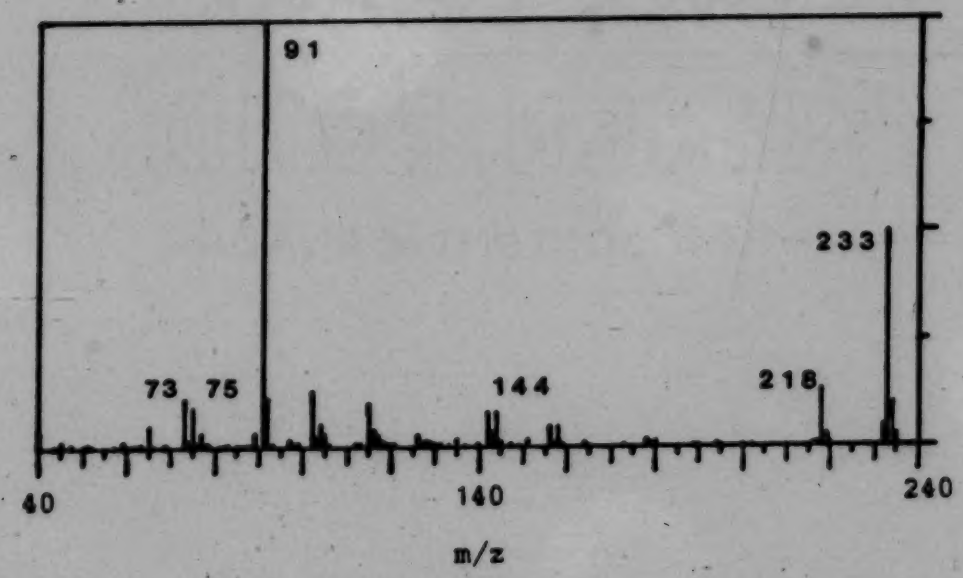


Figure 47 . Mass spectral fragmentation pattern of the BSA derivative of NOH-PBA.

structure was based on comparison of its GLC-MS properties with those of authentic NOH-PBA and its BSA derivative. Authentic NOH-PBA was obtained by the hydrogen peroxide oxidation of PBA.

Biotransformation studies on N-propargylbenzylamine (PBA, Fig. 45) were undertaken to provide insight into the metabolic sequence involved in the formation of the N-oxidation product, NOH-PBA. The substrate (PBA) was completely metabolized and only two products were recovered, the N-acetyl conjugate, AcBEN, and the major metabolite, NOH-PBA.

Propiolaldehyde and formaldehyde have been reported in mammalian systems as N-dealkylation products of N-propargyl and N-methyl substrates, respectively (Burckard *et al.* 1965; Savenije-Chapel and Noordhoek 1980; Shirota *et al.* 1980; Shirota *et al.* 1979). Attempts to detect the semicarbazide derivatives of these products in incubation broths to which semicarbazide reagent had been added were not successful.

The relative quantities of N-dealkylation products were decreased substantially when *C. echinulata* was incubated with PAR in 5% trypticase soy broth medium. PAR accounted for 82% of the total recoverable material. It is noteworthy that this medium caused a morphological change in *C. echinulata* from filamentous to micro pelleted cells.

Incubation of deprenyl (DEP) with *C. echinulata* resulted in the formation of four metabolites. Deprenyl was not detected in the extracts. The metabolites were

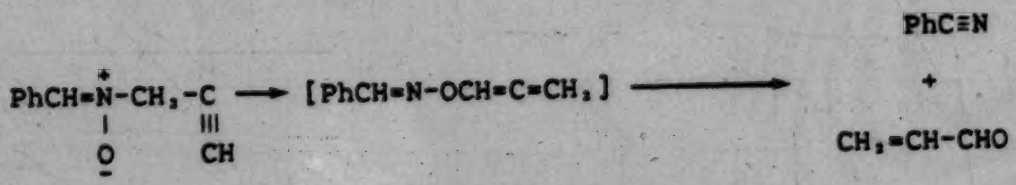
unequivocally identified as AMPH, NMA, OXM and AcAMPH (Table 15). Surprisingly the N-demethylation product of deprenyl, N-propargylamphetamine, was not detected as a metabolite. The corresponding product of pargyline metabolism, N-propargylbenzylamine, was the major metabolite.

The amount of added substrate had a significant influence on the percent recovery of pargyline metabolites after incubation for 168 h (Table 16). Only N-Propargylbenzylamine and the N-oxidation product, NOH-PBA were detected in samples containing 7.0 mg of substrate. Lower substrate levels (0.7 mg) favoured the formation of MBA. In contrast, changes in substrate concentration did not appear to play a major role in the biotransformation of deprenyl. The N-acetyl metabolites AcAMPH and AcBEN are stable conjugates indicative of the metabolic formation of the N-dealkylation products AMPH and BEN, respectively. The low recovery of AcAMPH from deprenyl incubates was surprising in that it was found to be a major metabolite of amphetamine. It is noteworthy that only two N-oxidation products NOH-PBA and OXM were isolated; it is unusual that conversion of the N-hydroxyl compound to the corresponding nitrone was not detected (Coutts and Beckett 1977). The absence of the nitrone, however, may be due to its thermal rearrangement via the *o*-allenyl oxime to acrolein and benzonitrile (Scheme 17) in a manner similar to that reported for the degradation of pargyline to acrolein and an imine (Hallstrom *et al*. 1980).

Table 16 . Effect of substrate concentration on the relative recovery of deprenyl and pargyline and their metabolites with *C. echinulata*'.

Biotransformation mechanism	Deprenyl		Pargyline	
	0.7mg	7.0mg	0.7mg	7.0mg
Recovered substrate	-	-	-	0.8
Didealkylation	61.1	40.5	-	12.6
N-acetylation	-	3.0	30.2	23.4
N-demethylation	-	-	-	49.6
N-depropargylation	38.9	56.5	69.8	7.1
N-oxidation	-	trace	-	6.5

'Amount of substrate added to 25mL of incubation medium.



Scheme 17

There are significant differences in the relative amounts of the recoverable products observed in the extracts of basified *C. echinulata* incubations of either deprenyl or pargyline. Substrate concentration is obviously important (Table 16), but other factors including lipophilicity of the substrate and its metabolites contribute to these variations. The overall lipophilicity of these substrates and their metabolites is influenced by the size and complexity of the N-alkyl side chain and degree of *alpha*-carbon substitution (Kubinyi 1979; Mihailova and Testa 1979; Siewinski *et al*. 1977). Other studies have found that mammalian drug metabolizing systems are influenced by the substrate concentration (Dial and Clarke 1979).

4.8.2 Comparison of Microbial and Mammalian Metabolism

Pargyline is metabolized by the rat to benzylamine, N-methylbenzylamine and N-propargylbenzylamine (DeMaster and Nagasawa 1978; Diehl *et al*. 1976; Durden *et al*. 1976; Edwards and Blau 1973; Parkinson and Callingham 1980; Shirota *et al*. 1979; Shirota *et al*. 1980; Valerino *et al*. 1978). N-methylbenzylamine has been recovered from human urine and was also present with benzylamine in human and rat liver homogenates (Pirisino *et al*. 1979). Amphetamine and

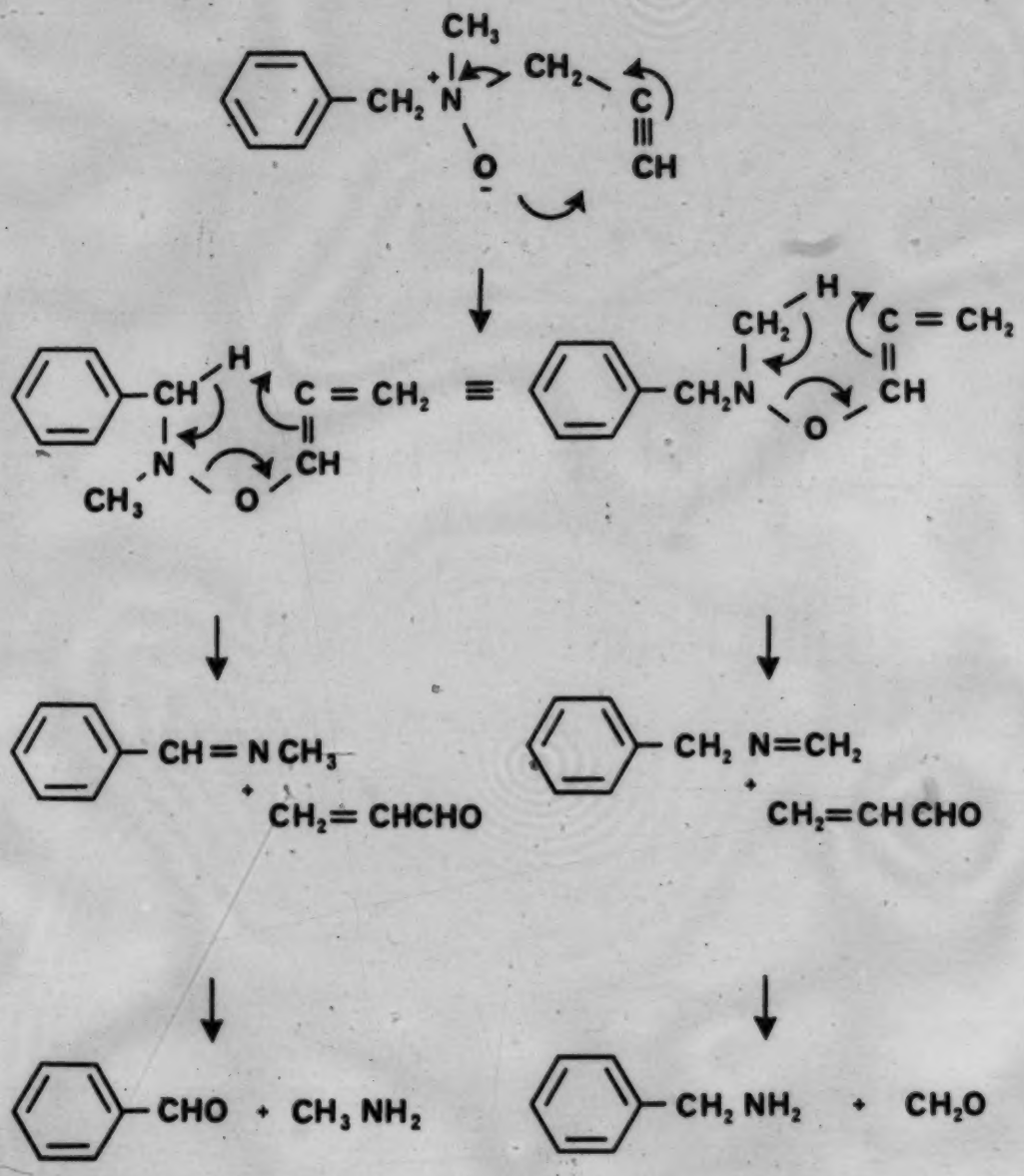
N-methylamphetamine were recovered from human and rat liver homogenates containing deprenyl (Reynolds *et al*. 1978; Reynolds *et al*. 1978a; Reynolds *et al*. 1979). Rat and rabbit liver microsomal preparations can metabolically N-oxidize pargyline to the N-oxide by two different routes. In rat, cytochrome P-450 is involved in the formation of pargyline N-oxide whereas in rabbit, N-oxidation is also mediated by an oxygen and NADPH dependent enzyme system similar to the amine oxidase described by Zeigler and Mitchell (1972). This Meisenheimer type rearrangement is involved in the decomposition of pargyline N-oxide (Fig. 48)(Hallstrom *et al*. 1980; Hallstrom *et al*. 1981). The Schiff bases may undergo hydrolysis to the respective aldehyde and the primary amines, benzylamine and methylamine.

4.9 Hydrazines

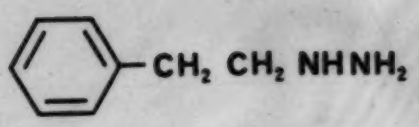
Hydrazines and hydrazides are both capable of reacting with natural aldehydes and ketones, pyridoxal phosphates, some monosaccharides and carbohydrates to form hydrazones and osozones.

In man and most laboratory animals, one of the most important biotransformations of the hydrazine group is acetylation of the terminal nitrogen group. It is interesting that in human populations polymorphism exists with the capability to acetylate hydralazine, phenelzine, some sulfonamides and other medicinal hydrazines by a

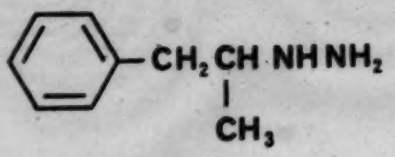
Figure 48 . Meisenheimer type rearrangement of pargyline N-oxide.



hepatic N-acetyltransferase (Juchau and Horita 1972; Tilstone *et al.* 1979). Some people are fast acetylators others are slow acetylators (Marshall 1976).

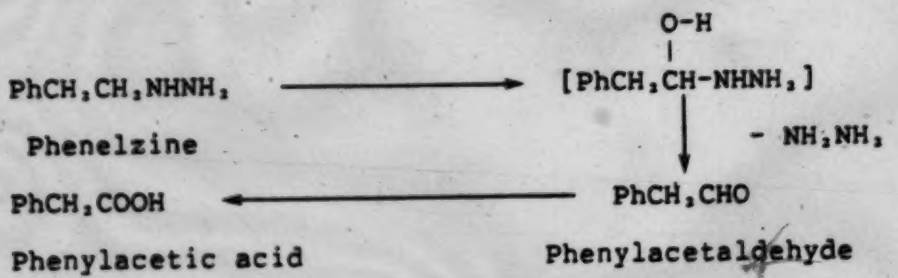


Phenelzine



Pheniprazine

The compounds phenelzine (Nardil, PLZ, (2-phenylethyl)hydrazine) and pheniprazine (Catron, PNZ, (2-phenylisopropyl)hydrazine) are structurally related to the phenylethylamine class of compounds. These compounds are used in the treatment of depression (Anderson *et al.* 1961; Evans *et al.* 1975). Phenelzine is rapidly oxidized to phenylacetic acid via the monoamine oxidase pathway (Scheme 18)(Clineschmidt and Horita 1969,b).



Scheme 18

Acetylphenelzine has not been demonstrated as an *in vivo* metabolite but transfer of ^{14}C-acetate from labelled acetyl CoA to phenelzine by N-acetyltransferase has been observed in *in vitro* human and rat liver systems (Tilstone *et al.*

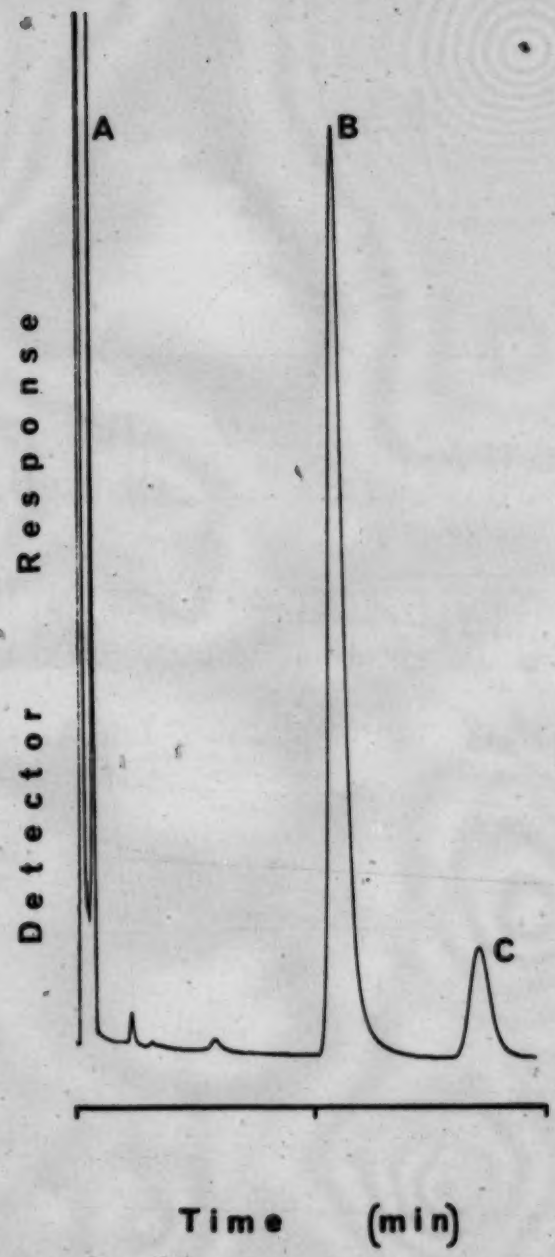
1979). The acetylphenelzine molecule obtained by this method has not been characterized by either GLC or GLC-MS techniques. Recent studies on the effects of PLZ on biogenic amines in the central nervous system have reported detecting trace amounts of PHE (Boulton *et al.* 1975; Dyck *et al.* 1981).

The metabolic pathway of pheniprazine is poorly understood although evidence indicates that this compound is rapidly inactivated by reaction with endogenous biological substances and N-N bond cleavage to amphetamine (Boulton *et al.* 1975). The major biotransformation route of hydrazines in man and laboratory animals is N-acetylation and dehydrazination.

Intestinal micro-organisms are capable of reductive fission of hydrazines but not hydrazides under anaerobic incubation conditions (Bolton and Griffiths 1978). *Pseudomonas sp.* PRL-F84, was found to cleave the N-N bond of 1,4,5,6-tetrahydro-6-oxo-3-pyridazinecarboxylic acid under aerobic conditions when this substrate was the sole source of carbon and nitrogen for growth of the organism (LaRue and Child 1979). Little is known however, about the aerobic transformation of hydrazines by micro-organisms.

Extracts of control and *M. smegmatis* incubation broths containing phenelzine (PLZ) were examined by GLC (Fig. 49). Three GLC peaks were observed using column B, (section 3.4.5); these peaks are referred to as A-C on the figure. Peak A corresponded to the substrate PLZ and peak B was

Figure 49 . GLC trace of the metabolic profile obtained from *M. smegmatis* biotransformation of phenelzine. System B, 145°C.



identified, by direct comparison with an authentic mass spectrum, as the N-acetyl derivative of phenylethylamine, AcPHE. The MS fragmentation pattern observed for peak C was consistent with that expected for the N-acetyl derivative AcPLZ, (Fig. 50). A portion of the original extract of the *M. smegmatis* incubation with PLZ was reacted with acetic anhydride and re-analysed. PLZ and the peak tentatively identified as AcPLZ were absent from the GLC trace and a new peak was concomitantly formed. This new peak was found to have GLC-MS properties identical to those of authentic N,N-diacetylphenelzine (Fig. 50). The position of N-mono-acetylation in the original *M. smegmatis* metabolite was not discernable from the MS fragmentation data. Two structures were possible and further characterization was required. Studies with TFAA have shown that the position of N-acetylation can be elucidated by the derivatization pattern observed. TFAA was allowed to react with a portion of the extract for 15 min. The solution was reduced to dryness, redissolved in ether-methylene chloride and analyzed by GLC/MS. A short retention time product was obtained which, on standing was converted into a longer retention compound. Both products had virtually identical mass spectra (Fig. 51). These observations are consistent with route (a) and are incompatible with route (b) which could only produce one TFA derivative (Fig. 52). Further confirmation of the inappropriateness of route b was obtained from studies with ethyl chloroformate. The

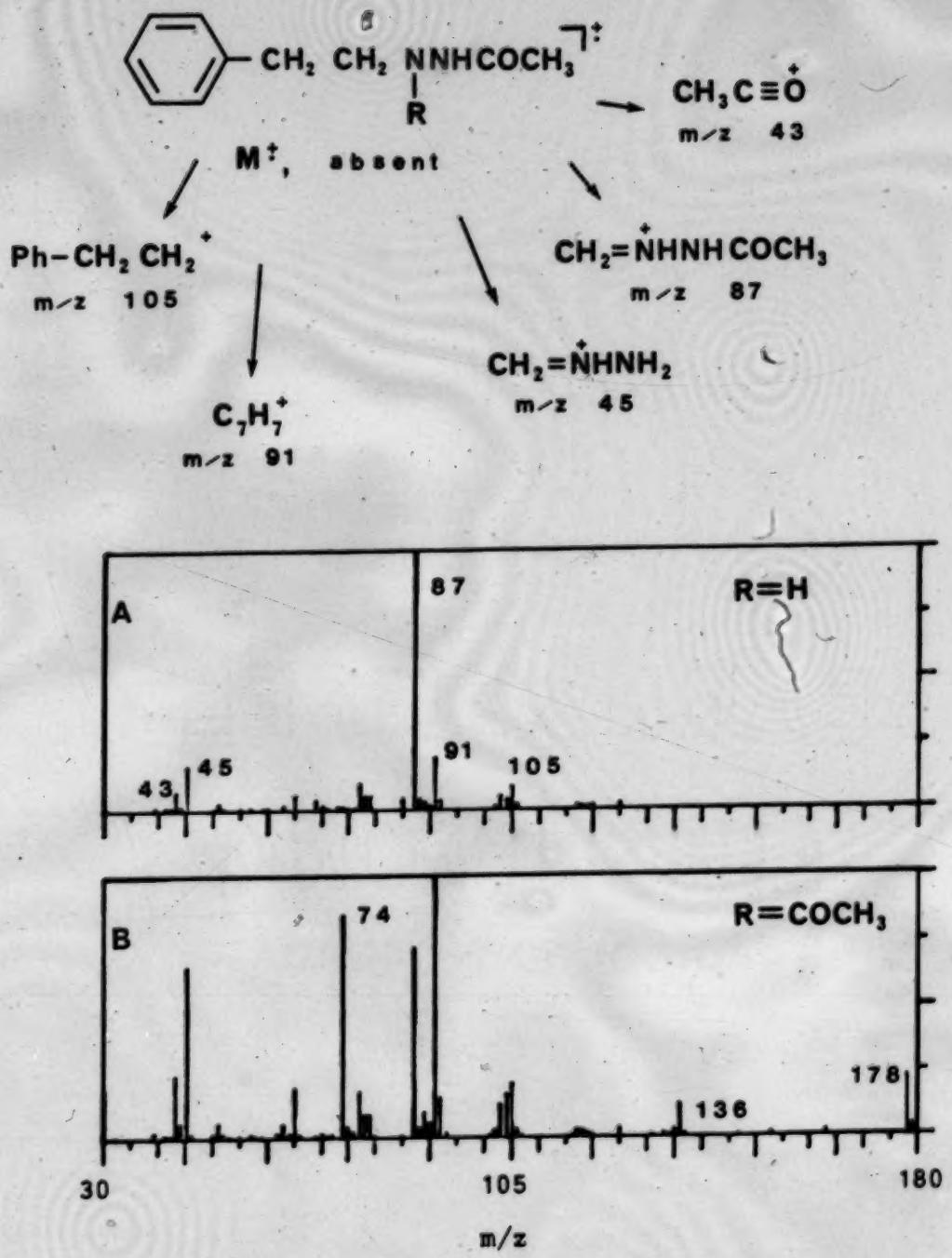


Figure 50 . Mass spectral fragmentation patterns of the acetylated derivatives of 1-acetyl-2-(2-phenylethyl)-hydrazine.

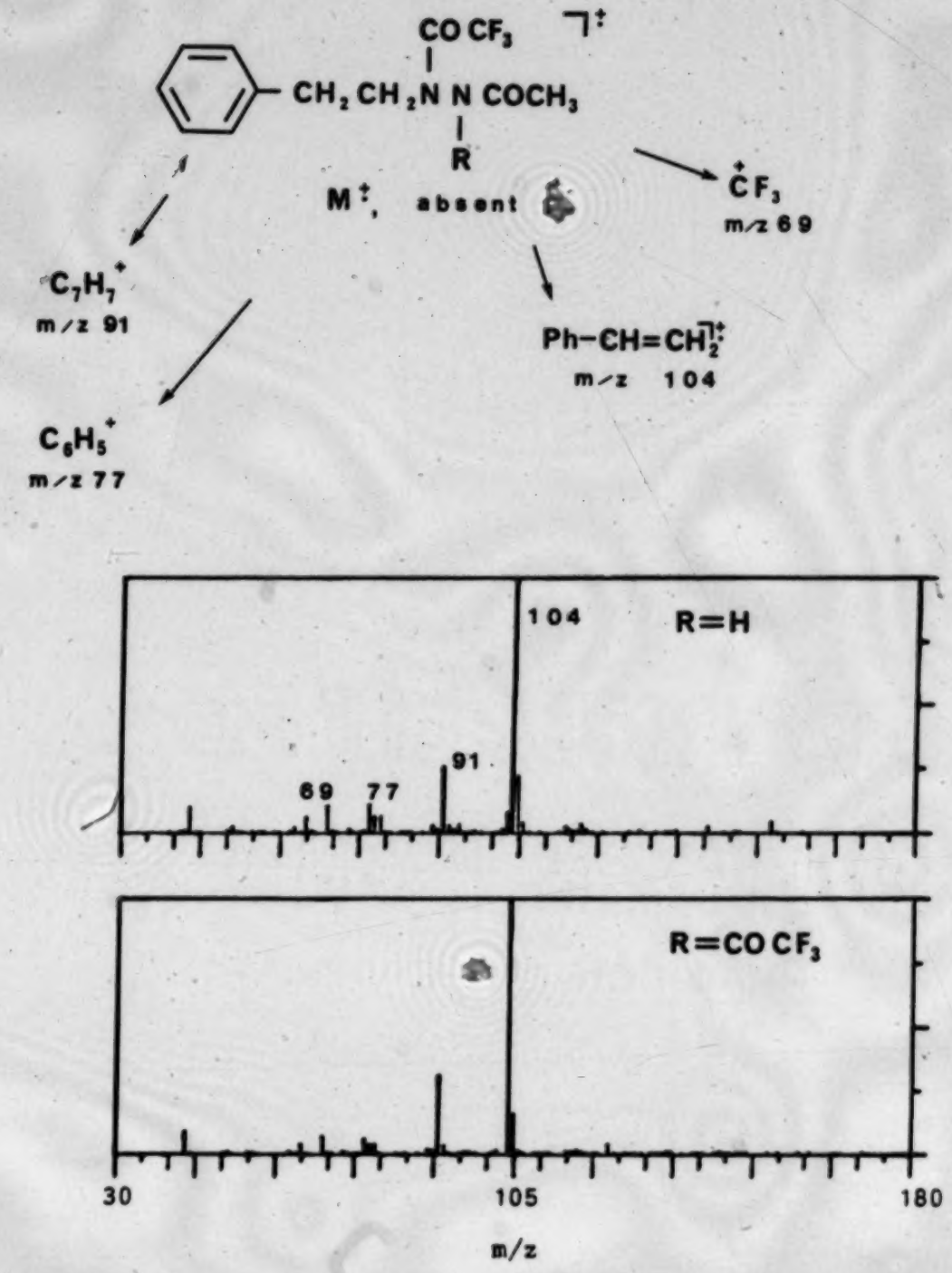
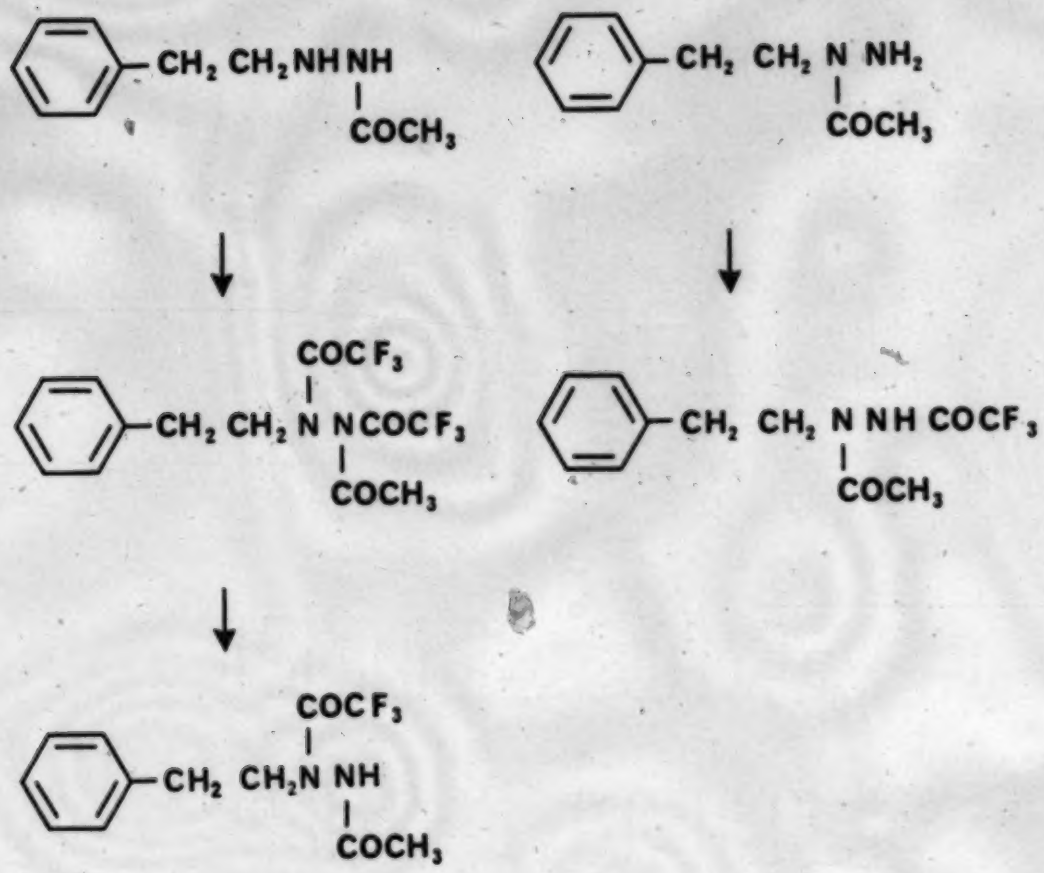


Figure 51 . Mass spectral fragmentation patterns of the trifluoroacetic acid anhydride derivatives of 1-acetyl-2-(2-phenylethyl)hydrazine.



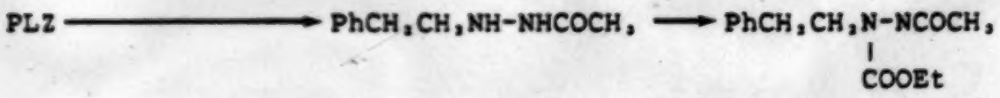
Route a Route b

Figure 52 . Expected trifluoroacetic acid anhydride derivatization patterns of AcPLZ.

ethylformate derivative of PLZ was reacted with acetic anhydride (Fig. 53) to produce the known monoacetate (Scheme 19).

Scheme 19

A portion of the extract from the metabolism of PLZ was reacted with ethyl chloroformate (Scheme 20).

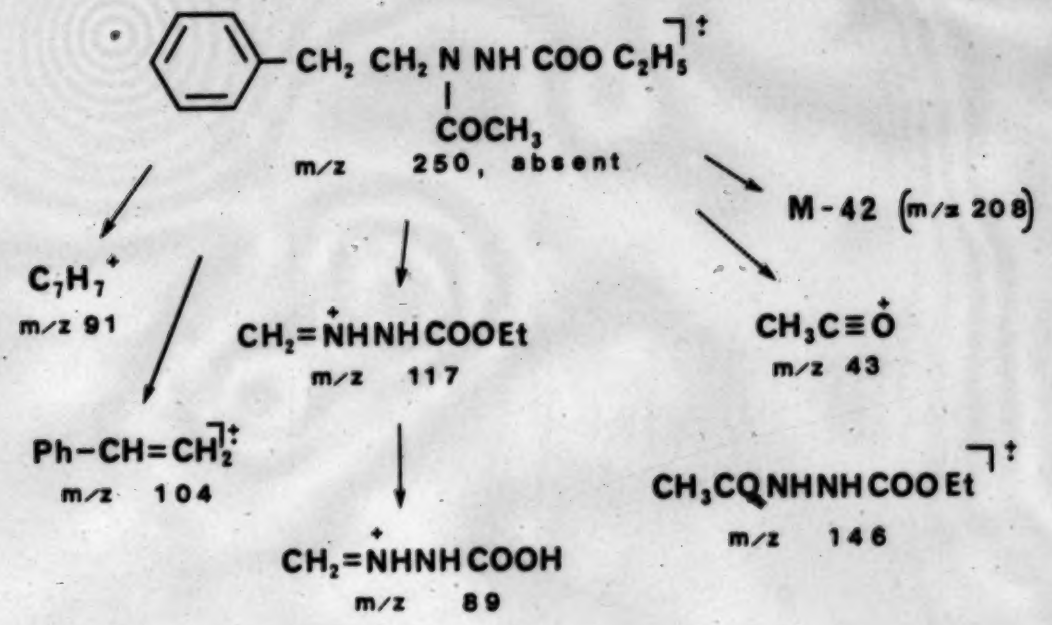


Scheme 20

The product obtained (Fig. 54), as expected, was not identical to that of the acetyl derivative of the chemically synthesized derivative. The isomeric structure was the only possible alternative.

Samples of the incubation broth were also extracted under acidic conditions (Method B, section 3.4.1). The chloroethanol derivative of phenylacetic acid was not detected. PLZ is the amino isostereomer of N-methylphenylethylamine. If phenylacetic acid was a metabolite of PLZ, it was completely utilized by the micro-organism.

Tilstone *et al*. (1979) speculated that the absence of AcPLZ in human biological fluids of patients receiving PLZ may be due to low plasma levels of PLZ. It is probable though that this metabolite has not been detected for a combination of reasons. The addition of the N-acetate grouping increases the lipophilicity of this compound



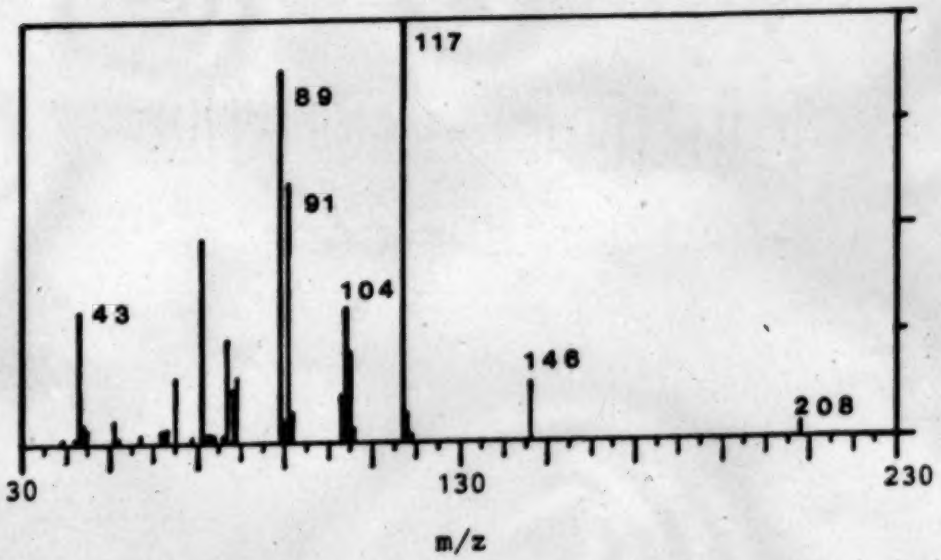
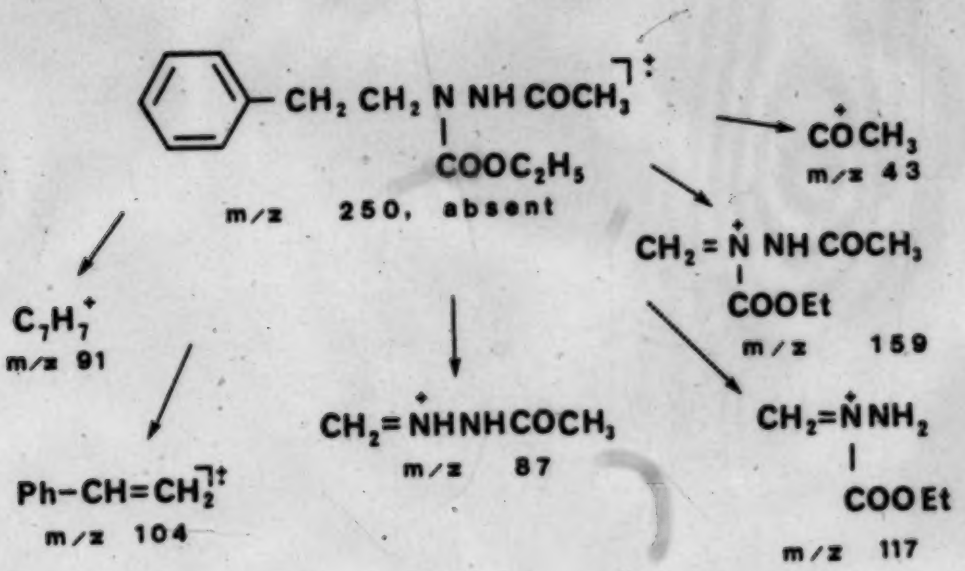


Figure 53 . The mass spectra and fragmentation pattern for the acetylated derivative of PEF.



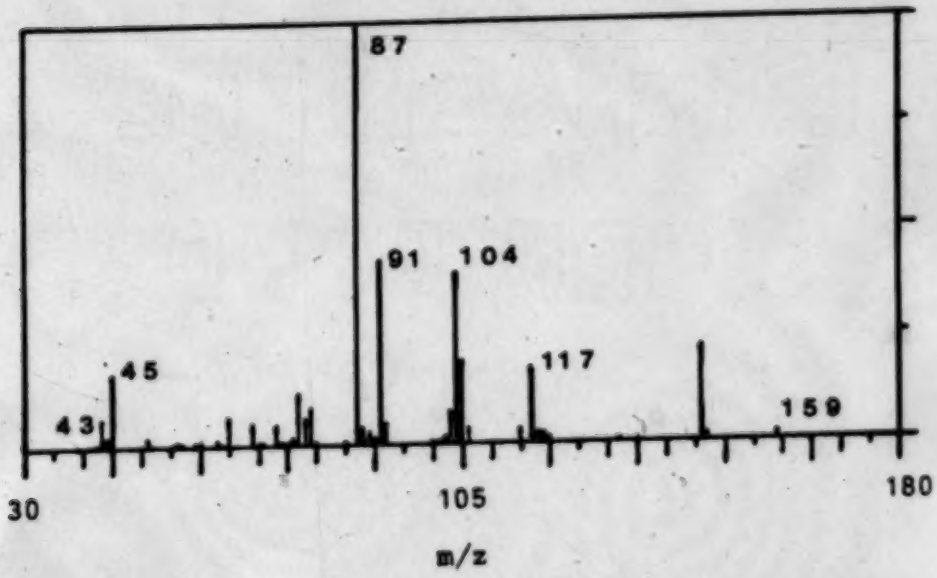


Figure 54 . Mass spectral properties of the product obtained by treating a portion of the original extract containing AcPLZ with ethyl chloroformate.

which would not only alter the excretion characteristics of the molecule but would also make it available for further transformations, such as dehydrazination. Alternatively the analytical systems may have not been adequate for the detection of AcPLZ in biological fluids.

In a similar manner, three compounds, were observed in the GLC traces of extracts obtained from *M. smegmatis* incubation broths containing pheniprazine (PNZ) (Fig. 55). They were identified as PNZ, AcAMPH and 1-acetyl-2-(1-methyl-2-phenylethyl)hydrazine (AcPNZ) (Fig. 56) by comparison of their GLC/MS properties with those of authentic samples.

Recovery of PLZ, PNZ and their metabolites was not complete due to the instability of these substrates in alkaline solution, probable reaction with media and microbial constituents and the possibility of alternative metabolic pathways. PNZ is known to auto-oxidize in solution to isopropylbenzene, phenylacetone and 1-phenyl-2-propanol, making quantitative recovery of this substrate difficult (Colvin 1969; Jonsson *et al*. 1977).

In contrast to the metabolic diversity of *M. smegmatis*, *C. echinulata* metabolism of PLZ and PNZ resulted in the formation of single products, AcPLZ and AcPNZ, respectively.

There were only two routes of transformation observed in this study on PLZ and PNZ: N-acetylation and N-N bond cleavage. The mechanism involved in the cleavage of this bond is unknown. On the basis of the studies with *M.*

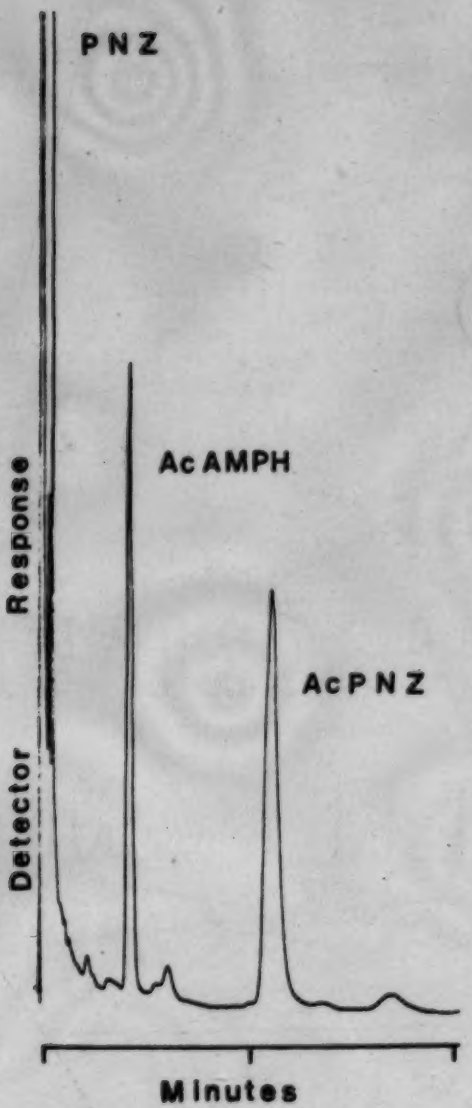
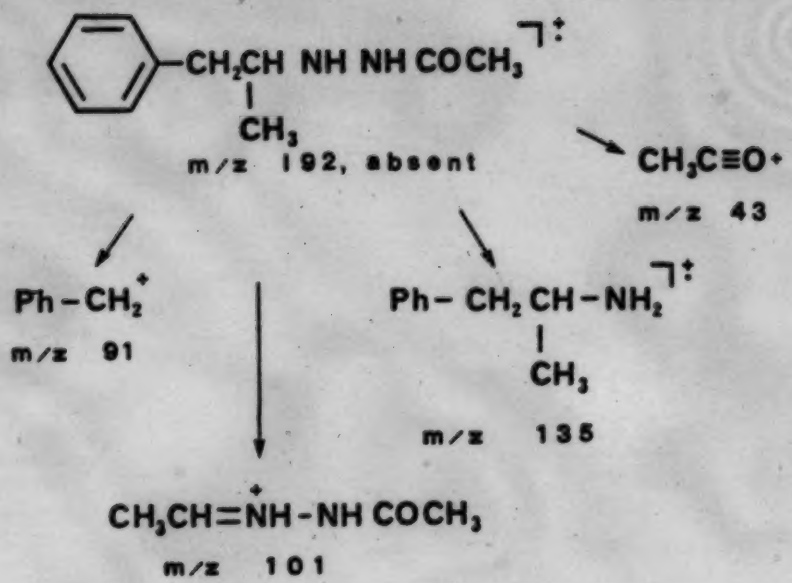


Figure 55 . GLC trace of pheniprazine metabolites formed by *M. smegmatis*. System B, 175°C.



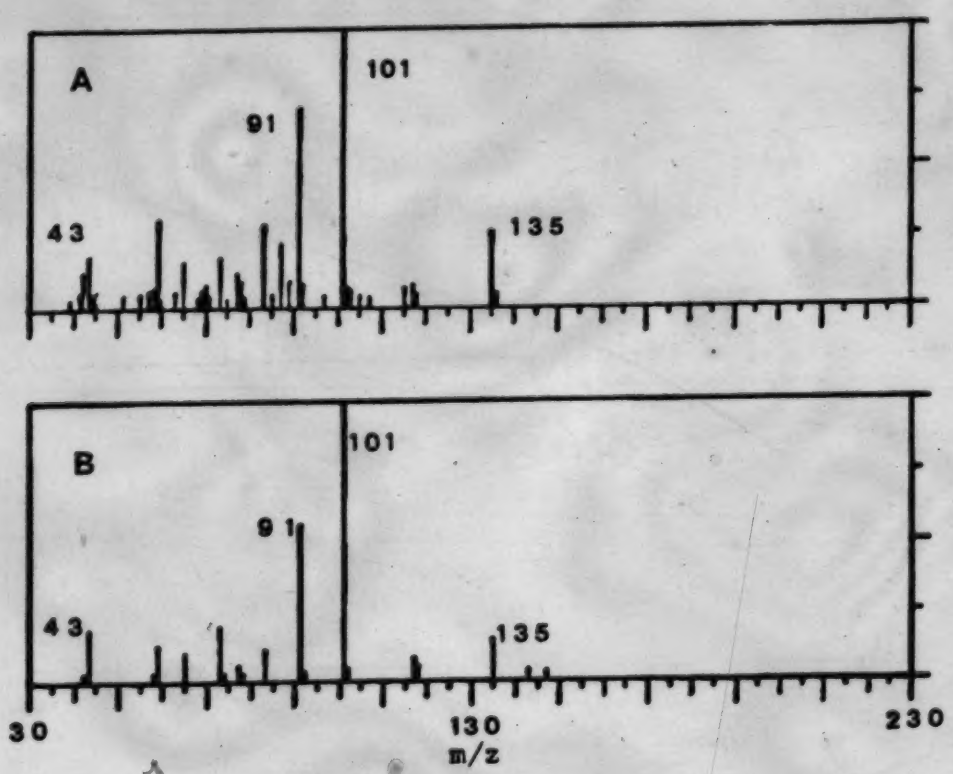
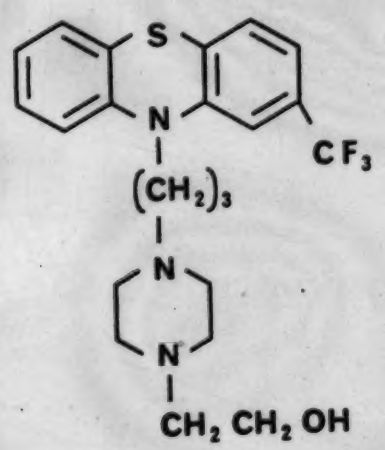


Figure 56 . The mass spectral properties of 1-acetyl-2-(1-methyl-2-phenylethyl)hydrazine (A) and the acetic anhydride derivative (B).

smegmatis described earlier (section 4.6) where N-oxidation was observed to be a major metabolic route, it can be speculated that the cleavage may be due to oxidation, reduction or a combination of both (Fig. 57). The oxidative reaction may involve formation of a labile N-hydroxylation intermediate. The incubation conditions were aerobic and if the mechanism is not oxidative, the amine must be formed by reduction with a oxido-reductase system (Kieslich 1978). It is also possible that the N-N bond cleavage was mediated by a extracellular hydrolase.

4.10 Fluphenazine



The investigations with *C. echinulata* and *M. smegmatis* so far described have shown that these organisms are able to transform a number of substrates by similar biotransformation routes, in particular by nitrogen (heteroatom) oxidation. Further investigation of heteroatom oxidation seemed warranted. Fluphenazine (FLU) was chosen as the

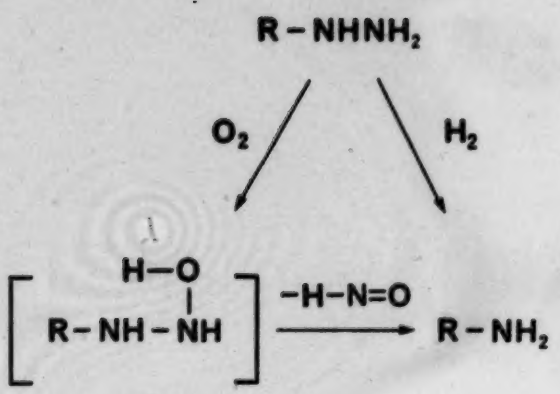


Figure 57 . The proposed routes for the metabolic N-N bond cleavage of phenelzine and pheniprazine by *M. smegmatis*.

substrate for this study. FLU is a tricyclic antipsychotic which is used in the treatment of schizophrenic-type disorders (Franklin *et al.* 1978). In liver microsomal preparations from the male rat, N-dealkylation, N-oxidation, S-oxidation and aromatic hydroxylation were the major routes of biotransformation (Gaertner *et al.* 1974). The relative rates of the reactions depended on the substrate concentration, N-oxidation being favoured at higher concentrations. The major *in vivo* metabolite of FLU in dogs and monkeys was shown to be 7-hydroxyfluphenazine (7OH-FLU) (Dreyfuss 1971). In addition, FLU sulfoxide (FSX) and an unknown metabolite were also detected. In the rat, FLU enanthate was metabolized to FLU and FSX and only free FLU was found in the brain following injection of FLU enanthate and decanoate. Flu and its principle metabolites FSX and 7OH-FLU were detected and quantified in human plasma, urine and faeces following intramuscular and oral adminstration of the HCl salt (Whelpton and Curry 1976). A conjugate fraction was also observed.

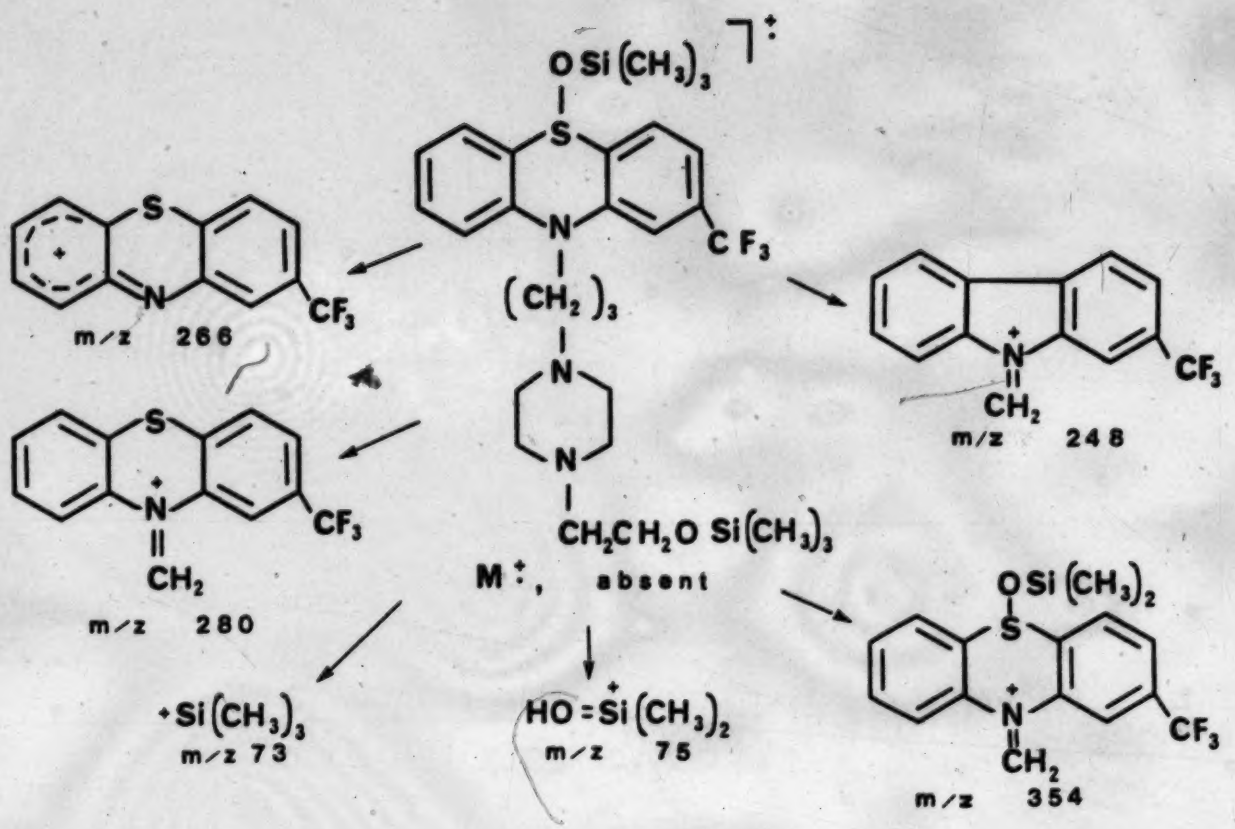
The alkaline extract (Method A, section 3.4.1) of *C. echinulata* (day 5) incubation broths containing either 0.35 mg/flask or 7.0 mg/flask of FLU were subjected to HPLC analysis after acetylation. UV analysis of the 7.0 mg/flask sample produced a different profile from that obtained by electrochemical analysis for this sample. In addition to FLU a second peak , not seen by electrochemical analysis, was detected at 8.0 min. This observation is consistent with

that expected for a ring hydroxylation product (personal communication with Dr. R.A. Locock). The HPLC profiles of the 0.35 mg/flask sample obtained from both UV and electrochemical analysis were identical. Comparison of HPLC retention times with that of an authentic standard indicated the metabolite present in this extract was FSX.

The incubation broth from which this sample (0.35 mg/flask) was obtained was extracted (Method A, section 3.4.1) and reacted with BSA. Comparison of GLC (column C) and GLC/MS properties of this extract with those of FSX treated in the same manner confirmed the metabolic formation of FSX (Fig. 58) by *C. echinulata*.

The GLC trace obtained for the extract from the day 7 incubation broth containing 0.35 mg/flask of FLU was similar to that observed on day 5. The GLC trace of the extract from the incubation broth containing 7.0 mg Flu revealed the presence of several additional peaks. Mass fragmentography of this sample for major diagnostic ions present in FLU established that these new compounds were metabolites of this substrate. No additional studies were carried out on this substrate.

M. smegmatis was not able to mediate biotransformation of FLU.



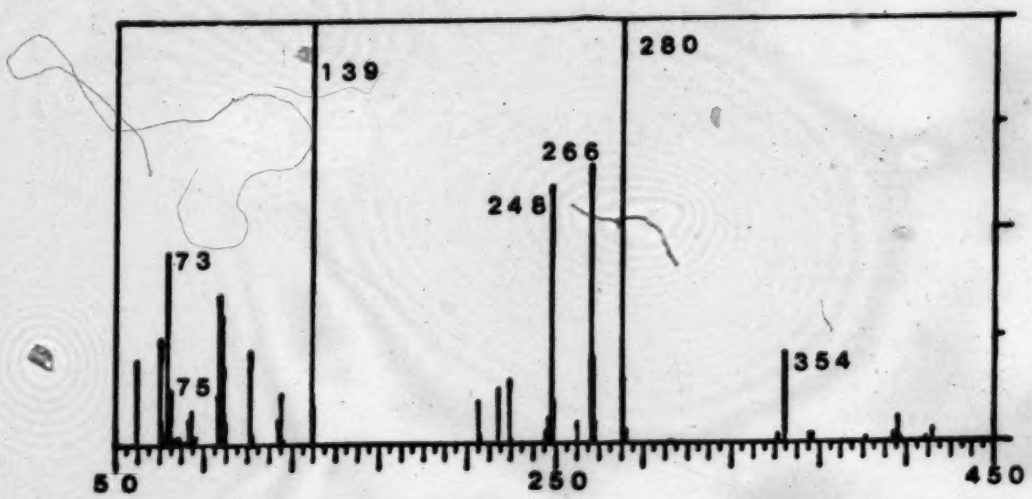


Figure 58 . Mass spectral fragmentation pattern of the TMS derivative of fluphenazine sulfoxide.

4.11 Microbial Synthetic Reactions

It was evident that *C. echinulata* and *M. smegmatis* were able to mediate the formation of novel metabolites, several of which are difficult to synthesize chemically. When using micro-organisms to synthesize scaled up quantities of products several factors must be considered: the organism used and the environmental conditions, the amount of active enzyme in the biomass and the length of the production phase.

Initiation of traditional batch reactors necessitates that the reaction vessel be refilled with medium, sterilized and inoculated with relatively few cells that must grow to establish the high biomass and correct physiological state for biotransformation of the substrate. The useable life of a microbial incubation in a batch reactor is governed by the accumulation of toxic products and the depletion of limiting substrates. Recent developments in microbial bioreactors has increased the usefulness of micro-organisms for the synthesis of important products.

Immobilized cells may display characteristics different from those observed in freely suspended cells (Abbott 1977; Abbott 1978; Jack and Zajic 1977). The specific advantage is dependant on the biotransformation and method of immobilization. Abbott (1977, 1978) listed several advantages which illustrates the benefits of immobilized cells. The major advantages are that the process can be made continuous, using a higher biomass than that generally found

in free cell systems due to modified rheological properties of the bioreactor as a result of immobilization to provide a saving of both time and expense.

4.11.1 Immobilized Microbial Cells

Carrageenan from two suppliers (Sigma and FMC Corporation) were tested for their ability to support microbial growth and influence on biotransformation (Chibata and Tosa 1980; Tosa *et al.* 1979).

4.11.1.1 Sigma Carrageenan

The beads prepared with this product were relatively free of visible *C. echinulata* biomass initially and only a few beads changed from translucent to opaque with time in the batch reactor. It was evident during microscopical analysis, aided by pretreating the beads in a dilute solution of methylene blue, that the filamentous organism extended throughout the opaque beads but was virtually absent in the translucent beads. Biotransformation studies with NEA, NPA and PLZ and carrageenan immobilized *C. echinulata* were partially successful as known metabolites were produced; however, the quantities detected were relatively minor compared with free cell systems.

This may in part be due to the temperature required for the bead extrusion process and use of glutaraldehyde as a stabilizer. These factors may have in fact damaged essential enzymes, preventing the organism from achieving its

characteristic growth and biotransformation pattern. It is likely that the carrageenan used was unsuitable for cell immobilization.

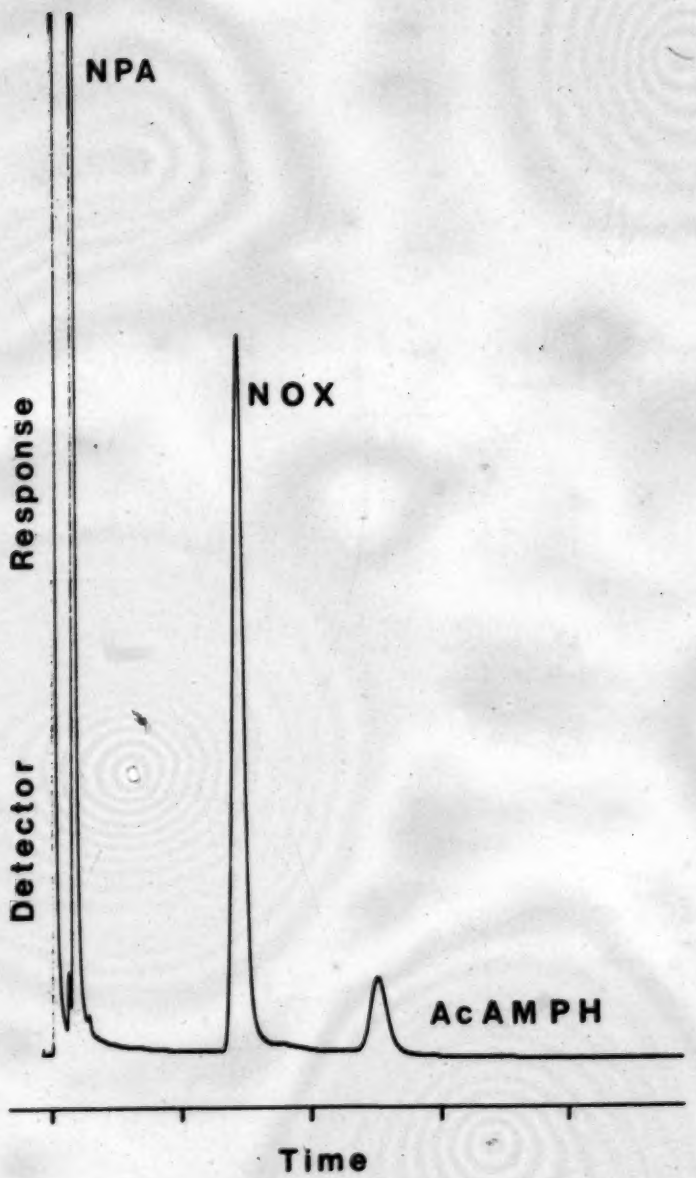
4.11.1.2 FMC Carrageenan

C. echinulata immobilized in FMC carrageenan NJAL 724 was able to grow rapidly within this gel matrix and all beads were opaque when observed at 48 h. Microscopic analysis revealed that the beads were heavily grown in the bead and on the surface with this organism. Extension of the fungal biomass into the medium was a severe problem in the batch reactor as shearing forces resulting in free cells which increased the apparent viscosity of the medium to that comparable with free cell systems. Biotransformation studies with NEA, NPA and PLZ using this reactor were productive and indicated that all metabolic routes were available with N-oxidation and N-acetylation predominating.

High levels of biotransformation were observed when immobilized cells were incubated in the fluidized bed reactor with NPA. In contrast to earlier studies with NPA, a single major product, NOX, was obtained with only minor quantities of AcAMPH (Fig. 59). This conversion was in the order of 5-7 fold greater than that generally observed in free cell systems.

Immobilized *C. echinulata* incubated in the air lift reactor was able to completely acetylate PLZ to AcPLZ within 24 h. Supplementing the reactor with PLZ over a three day

Figure 59 . GLC trace of the extract obtained from a fluidized bed reactor packed with immobilized *C. echinulata* containing NPA as substrate. System B, 145°C.



period allowed for continued production of AcPLZ, the reactor was stopped at this time only as a result of contamination.

Immobilization facilitated the use of filamentous fungi by altering the apparent rheological properties of the medium. Fluid viscosity was decreased and this provided better mixing and mass transfer of nutrients, gases and substrate. In addition the interaction of organic compounds with the solid-liquid interface of the beads may result in enrichment of the micro-environment to give substrate concentrations higher than that available in the bulk of the medium (Abbott 1977). This factor and higher hydraulic rates gave immobilized *C. echinulata* higher productivity rates than those obtained in the batch reactor with freely suspended cells.

4.11.2 Air Lift Reactor Production of Microbial Metabolites

The apparent benefits of the air lift reactor for the production of metabolites with these substrates suggested that the system may be useful for the production of antibiotics and other important microbial products. *Trichodera sporulosum* was chosen as a model organism for this study. *T. sporulosum* was incubated in the air-lift reactor as a free cell suspension. Complete mixing of the reactor was accomplished by increasing the oxygen supply when necessary. Portions of the incubation broth were tested for anti-microbial activity (Table 17). A swarming effect

Table 17 . Bioassay results obtained for samples taken from an air lift reactor containing *T. sporulosum*.

	Day (zone of inhibition in mm)			
	5	7	9	13
Neurospora sitophilla	-	-	-	-
Proteus morganii	-	-	25	25
Rhodotorula glutinis	-	-	-	-
Salmonella typhimurium	25	35	35	22

was observed with the test organism *Proteus morganii* B23 at days 5 and 7. It was not until day 9 that antimicrobial activity was observed against this organism. The activity was enhanced when the incubation medium was treated with sodium bicarbonate prior to the bioassay. No activity was evident against *Neurospora sitophila* and *Rhodotorula glutinis*. The pH of the trypticase soy broth medium was initially 7.2 but by day 7 was between 8 and 9. Solid potassium phosphate dihydrate was added at this time to lower the pH to between 4.5 and 5.5, where it remained constant for the duration of the study.

The production of antimicrobial activity in the air lift reactor was compared with the production of antimicrobial activity in a shake flask. There was a major difference between the two reactors as the contents of the shake flask exhibited antibacterial activity and in addition expressed antifungal activity which was not apparent in the samples examined from the air lift reactor. The reactor contents (approximately 175 mL) were extracted twice with an equal volume of ethyl acetate. The solvent extracts were reduced in volume to about 2 mL and analyzed by TLC (solvent systems C-E, section 3.4.8). Four spots were detected after visualization with iodine using solvent E at Rf 0.07, 0.35, 0.63 and 0.78. Further studies are required to determine which of these spots represents the antimicrobial agent.

5. Summary and Conclusions

To establish the feasibility of using microbial models of mammalian drug metabolism required the examination of a series of structurally related compounds. This has been accomplished by studying the metabolism of amphetamine-like compounds by two organisms. The organisms were chosen from a wide variety of bacteria and fungi which were used initially to metabolize the model compound N-(*n*-propyl)amphetamine (NPA). NPA was selected as the initial substrate because it possessed a simple structure and yet was capable of undergoing a multiplicity of biological transformations. Caldwell (1976) identified six routes of biotransformation of N-alkyl amphetamines:

1. Aromatic hydroxylation in the ring 2, 3, or 4 positions with the possibility of dihydroxylation,
2. Aliphatic hydroxylation of the carbon atoms on the N-alkyl side chain,
3. N-dealkylation of the N-alkyl side chain,
4. Oxidative deamination to a ketone which could be further transformed to the respective alcohol or acid,
5. N-oxidation to produce hydroxylamines, oximes and nitroalkanes, and
6. Conjugation of amino, phenolic and alcoholic metabolites.

All of these metabolic routes have been demonstrated in animal systems, although in some instances the occurence of a particular route is comparatively minor (Caldwell 1976;

Vree and Walkenstein 1972).

The large number of available amphetamine analogues, and reports concerning their metabolites, lead to the selection of three substrate subgroupings:

1. Phenylethylamine, amphetamine, and phentermine and their ring or N-substituted analogues,
2. Hydrazine analogues of phenylethylamine and amphetamine, and
3. Tertiary amines containing a N-propynylic group.

Identification of microbial metabolites was confirmed by direct comparison of their GLC and GLC/MS properties with those of authentic reference compounds. In some instances, identification of a metabolite or its chemical derivative was based on interpretation of MS fragmentation patterns.

Having examined the microbial biotransformation of a wide range of amphetamine-like compounds, it was possible to delineate several general metabolism patterns. Ring hydroxylation was of little importance in the biotransformation of the phenylethylamines examined as well as in amphetamine metabolism. In general, this transformation was of major significance with the more lipophilic substrates examined, i.e. N-alkylated substrates and phentermine. This finding is in agreement with that of Coutts, Dawson *et al.* (1976). Oxidative deamination occured where permitted by substrate structure but the extent of cleavage was variable and strongly influenced by the presence of an *alpha*-carbon substituent. N-Dealkylation, was generally extensive. It is

noteworthy that in *M. smegmatis* biotransformation studies of N-alkyl amphetamines and phentermines, the extent of N-dealkylation was directly related to the degree of N-oxidation. This observation is supportive of a dealkylation mechanism described by Caldwell (1976) in which the N-alkyl substrate is initially N-oxidized and the product rearranges to a carbinolamine which subsequently dissociates to a primary amine and an aldehyde.

N-Oxidation was a major route of biotransformation in both *C. echinulata* and *M. smegmatis*. Classical inhibitors of mammalian cytochrome P-450, i.e. DCIP, carbon monoxide, potassium cyanide and SKF-525A, markedly reduced metabolic N-oxidation of NPA in *M. smegmatis* washed cell preparations. Two mechanisms of N-oxidation involving either a N-hydroperoxide or hydroxylamine intermediate were consistent with these findings. Further studies are required in order to determine whether the apparent differences in *C. echinulata* and *M. smegmatis* N-oxidation reactions are mediated by cytochrome P-450-like system or by a different enzymatic mechanism.

Although the formation of 1-phenyl-2-propanone oxime from N-hydroxyamphetamine has generally been regarded as a chemical process, it was shown that *M. smegmatis* could metabolically synthesize the oxime and that the biological product (the syn oxime) and chemical product (the anti oxime) could be differentiated by HPLC.

The biotransformation of amphetamine-like compounds by *C. echinulata* was similar to that observed in man with the formation of numerous metabolites, including products of oxidative deamination, N-dealkylation, N-oxidation and ring hydroxylation. Biotransformation studies lead to the identification of numerous acetylated products and N- and *p*-hydroxylated metabolites which have not been previously described. N-Acetylamphetamine has been suspected to be a metabolite of amphetamine in the rat (Dring *et al.* 1972); however, the identification of this product as a metabolite was not established unequivocally until this study.

Species variation was evident when *C. echinulata* metabolism was compared to that of *M. smegmatis*. *M. smegmatis* was unable to mediate ring hydroxylation of the substrates examined. However, in addition to the novel metabolites detected in *C. echinulata* incubation broths, *M. smegmatis* synthesized various compounds which have not been previously reported in mammalian studies.

It can be concluded that *C. echinulata* effectively mimics mammalian drug metabolism and *M. smegmatis* is a useful organism for mediating certain biological syntheses which are difficult to perform chemically.

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7. Curriculum Vitae

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1. Coutts, R.T., Foster, B.C., Jones, G.R., and Myers, G.E. 1979. Metabolism of N-(n-propyl)amphetamine by *Cunninghamella echinulata*. Appl. Environ. Microbiol. 37: 429-432.
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